III. REMARKS

Preliminary Remarks

Reconsideration and allowance of the present application based on the foregoing amendment and following remarks are respectfully requested. Claims 35-43 are currently pending and remain at issue.

In paragraph 5 of the official action, the examiner alleged the applicants have failed to identify nucleotide sequences of at least 10 nucleotides and amino acid sequences of at least 4 amino acids in the specification by a proper sequence identifier according to 37 C.F.R. §§ 1.821 though 1.825. In particular, on page 18, lines 11 and 13 of the specification, two oligonucleotide sequences *pox*Bint1 and *pox*Bint2 lack SEQ ID NOS.. Copies of the Sequence Listing in paper form and computer readable form for the above-identified application are attached hereto, in compliance with 37 C.F.R. §§ 1.821-1.825.

Pursuant to 37 C.F.R. §1.821(f) and (g), the applicants, through the undersigned attorney, hereby state that the sequence listing information of the attached copies of the Sequence Listing in paper and computer readable form are the same and do not contain new matter.

In paragraph 6 of the official action, the examiner objected to the abstract because it was allegedly not in a single paragraph. The applicants have amended the abstract and formatted it into a single paragraph.

New claim 44 is directed to an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of (a) a nucleotide sequence as set forth in SEQ ID NO: 1; (b) a nucleotide sequence encoding the polypeptide as set forth in SEQ ID NO: 2; and (c) a nucleotide sequence complementary to (a) or (b). New claims 45 and 46 are directed to the vector and host cells harboring the nucleic acid molecule of claim 44. Support for new claims 44-46 can be found throughout the specification, for example, on page 3, line 10 to page 4, line 12; and page 17, lines 31-35.

New claim 47 is directed to an isolated nucleic acid of claim 44 or a fragment thereof that encodes a polypeptide that has pyruvate oxidase activity. New claims 48 and 49 are directed to the vector and host cells harboring the nucleic acid molecule of claim 47. Support

for new claims 47-49 can be found throughout the specification, for example, on page 4, lines 13-20; and page 8, lines 3-5.

New claim 50 is directed to an isolated nucleic acid molecule that encodes a polypeptide that has the activity of pyruvate oxidase and hybridizes to the complement of the nucleic acid molecule of claim 42 under the following stringent conditions: a final wash of 0.5X SSC and 0.1% SDS at 68°C. Claims 51 and 52 are directed to the vector and host cells harboring the nucleic acid molecule of claim 50. Support for new claims 50-52 can be found throughout the specification, for example, from page 8, line 23 to page 9, lines 15; and page 19, lines 28-34.

The applicants do not intend by these or any amendments to abandon subject matter of the claims as originally filed or later presented, and reserve the right to pursue such subject matter in continuing applications.

Patentability Remarks

Rejection Under 35 U.S.C. §112, Second Paragraph

In paragraph 7 of the official action, the examiner rejected claims 41-43 under 35 U.S.C. §112, second paragraph, as being indefinite. Specifically, the examiner alleged claim 41 is unclear in the recitation of "[a] second isolated polynucleotide" because it is difficult to identify which polynucleotide is intended as being a first isolated polynucleotide. The examiner further asserted claims 42-43 are indefinite for the recitation of "having a sequence identical to a segment."

Amended claim 41 is now directed to a nucleotide sequence completely complementary to the isolated polynucleotide of any one of claims 35-37. In view of the foregoing amendment, the applicants submit that that the recitation "a second isolated polynucleotide" has been removed from claim 41 and therefore the indefiniteness rejection to this claim is now moot.

Solely for the purpose of expediting prosecution, and without prejudice to the applicants' right to seek broader claims in a continuing application, the applicants have canceled claim 42 without prejudice, thereby obviating the rejection of this claim. Amended claim 43 is now directed to an isolated oligonucleotide consisting of a fragment of 15-50

contiguous nucleotides of SEQ ID NO: 1. The applicants submit that the phrase "having a sequence identical to a segment" has been removed from claim 43, and therefore the indefiniteness rejection to this claim is now moot. In view of the foregoing amendments, the applicants submit that the rejection of claims 41-43 under 35 U.S.C. § 112, second paragraph, for being indefinite, has been overcome and should be withdrawn.

Rejections Under 35 U.S.C. §112, First Paragraph

Written Description

In paragraph 8 of the official action, the examiner rejected claims 42 and 43 under 35 U.S.C. §112, first paragraph, for lacking proper written description. Specifically, the examiner alleged that the specification describes only a single representative species of the claimed genus, *i.e.*, an oligonucleotide consisting of a fragment of 15-50 contiguous nucleotides of SEQ ID NO: 1. The examiner concluded the specification fails to describe any additional representative species of the claimed genus.

As discussed above, claim 42 has been canceled without prejudice. Similarly, claim 43 has been amended to be directed to an isolated oligonucleotide consisting of a fragment of 15-50 contiguous nucleotides of SEQ ID NO: 1. The applicants submit that one of skill in the art could use the sequence set forth in SEQ ID NO: 1 and choose 15-50 contiguous nucleotides to be used as a probe or as a PCR primer of the *pox*B gene of *Corynebacterium glutamicum*. Example 3 describes two such oligonucleotides *pox*Bint1 and *pox*Bint2. Accordingly, the applicants submit that due to the disclosure of the *C. glutamicum pox*B gene sequence as set forth in SEQ ID NO: 1 and the description of oligonucleotides in Example 3, the specification sufficiently describes the claimed invention in full, clear, concise and exact terms so that one of skill would recognize that the applicants were in possession of the claimed invention. In view of the foregoing amendment and remarks, the applicants respectfully submit the rejection of claims 42 and 43 under 35 U.S.C. §112, first paragraph, for lack of proper written description, has been overcome and should be withdrawn.

Enablement

In paragraph 9 of the official action, the examiner rejected claims 36 and 42-43 under 35 U.S.C. §112, first paragraph, for lack of enablement. Specifically, the examiner alleged that while being enabling for a nucleic acid encoding SEQ ID NO:2, including SEQ ID NO:1

and an oligonucleotide consisting of 15-50 contiguous nucleotides of a nucleic acid encoding SEQ ID NO:1, the specification does not reasonably provide enablement for the broad scope of claimed nucleic acids. The examiner also asserted that the specification fails to provide guidance regarding those nucleotides of SEQ ID NO: 1 that may be altered by substitution, addition, insertion, and/or deletion with an expectation of encoding a protein maintaining the desired activity. The examiner also alleged that while methods of generating variants of a given nucleic acid or oligonucleotide are known, it is not routine in the art to screen for all nucleic acids or oligonucleotides having a substantial number of modifications for those having the desired utility, as encompassed by the instant claims. The examiner concluded that without sufficient guidance, determination of having the desired biological characteristics is unpredictable, and the experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue.

As discussed above, claim 42 has been canceled without prejudice. Similarly, claim 43 has been amended to be directed to an isolated oligonucleotide consisting of a fragment of 15-50 contiguous nucleotides of SEQ ID NO: 1, which has been acknowledged by the examiner as fully described by the specification.

With regard to claim 36 and new claim 50, the issue of enablement involves the question of whether an application enables one of ordinary skill in the art to make and use the claimed invention. Experimentation is limited in claim 36 to an isolated polynucleotide, which encodes a polypeptide that is at least 95% identical to SEQ ID NO: 2, and wherein said polypeptide has pyruvate oxidase activity (see page 5, lines 12-17). Therefore, there is no question that the variants encompassed by the claims must retain the utility of the DNA sequence discovered and claimed by the applicants, and subsequently, allowed by the Patent Office. The applicants further submit that the specification enables one of skill in the art to make and functionally define the claimed variants, and methods of generating variant polynucleotides are known in the art as acknowledged by the examiner on page 9, lines 8 and 9 (see also page 10 of the specification).

Specifically, claim 36 satisfies the "how to make" prong of the enablement requirement because the scope of the claims are "reasonably correlated" with the teachings in the application [See MPEP §2164.01(b)]. The application and ordinary skill permit one skilled in the art to make any polynucleotides having 95% or greater sequence identity to the amino acid sequences recited in the claims by using PCR technology or other mutagenesis

techniques discussed on page 1, lines 22-27; page 5, lines 12-17; and page 9, line 23 to page 11, line 2. In fact, the Patent Office's own written description training materials acknowledge that "procedure[s] for making variants of [a protein having] SEQ ID NO: [3] which have 95% identity to SEQ ID NO: [3] and retain its enzymatic activity are conventional in the art." (See Revised Interim Written Description Guidelines Training Material, Example 14). Moreover, the application provides guidance as to the types of changes (*e.g.*, conservative mutations) that are more likely to retain functionality (see specification at page 10, lines 15-30.

The applicants submit new claim 50 also satisfies the "how to make prong" of the enablement requirement as well. New claim 50 is directed to hybridization variants under the stringent wash conditions of 68°C in 0.5X SSC and 0.1%SDS. Page 9, lines 4-15 and page 19, lines 28-34 provide methods for identifying *pox*B variant nucleic acids, which hybridize under stringent conditions to the complement of SEQ ID NO: 1. Further, the specification defines these highly stringent hybridization conditions (*i.e*, wash and hybridize at 68°C; see page 19, lines 30-34) and provide a reference for one of skill in the art to refer to *e.g.*, "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993)(*See Attachment A*).

With regard to the second requirement, the variants encompassed by amended claims 36, and new claim 50 are also functionally defined in that the claimed variants are limited to those that are capable of pyruvate oxidase activity. Namely, pyruvate oxidase in *Corynebacterium glutamicum* is a peripheral membrae flavoprotein that catalyzes the oxidative decarboxylation of pyruvate to yield acetate, CO_2, and reduced flavin adenine dinucleotide (pyruvate + FAD + Pi (TPP-thiamine pyrophosphate) → acetate + CO_2 + $FADH_2$). Identifying those encoded PoxB polypeptide variants recited in the claims can be achieved by routine screening assay that was well know to one of skill in the art at the time of filing (*See Attachment B*—Chang *et al., J. of Bact.* 167:312-318:313 (1986)). Since the pyruvate oxidase assay is routine in the art, one of skill could perform this assay without undue experimentation to identify 95% or higher variants encoding an amino acid sequence set forth in SEQ ID NO: 2, or hybridization variants of SEQ ID NO: 2 with pyrvuate oxidase activity. For example, the assay mixture to determine pyruvate oxidase activity of crude extracts of cells consists of sodium phosphate buffer, magnesium chloride, sodium thiamine pyrophosphate (phosphate source), and sodium pyruvate. The mixture is incubated at room

temperature with cell extracts and then $Na_2F_3(CN)_6$ as the electron acceptor is added to the mixture, and the rate of decrease of the absorbance of 450 nm is recorded. The enzymatic activity is calculated by using the formula $E_{450nm} = 218^{cm-1}$ and is expressed as the amount of pyruvate consumed per unit of time, assuming 2 equivalents of ferricyanide are reduced per equivalent of pyruvate decarboxylated. The pyruvate oxidase assay is performed using a spectrophotometer to detect changes in UV absorption in the assay. (*See Attachment B*, Chang *et al., J. of Bact.* 151:1279-1289, 1282 (1982); and Change *et al., J. of Bact.* 167:312-318, 313 (1986)). Accordingly, claims 36, 43, and new claim 50 are enabled.

In view of the foregoing amendments and remarks, the applicants submit that the rejection of claims 36, 42, and 43 under 35 U.S.C. §112, first paragraph, for lack of enablement, has been overcome and should be withdrawn, and of new claims 50-52 should not be rejected upon the same grounds.

IV. CONCLUSION

In view of the foregoing, the claims are now believed to be in form for allowance, and such action such action is hereby solicited. If any point remains in issue which the examiner feels may be best resolved through a personal or telephone interview, please contact the undersigned at the telephone number listed below.

Respectfully submitted,

PILLSBURY WINTHROP LLP

By: _____

Thomas A. Cawley, Jr., Ph.D.
Reg. No. 40944
Tel. No. (703) 905-2144
Fax No. (703) 905-2500

TAC/PAJ
P.O. Box 10500
McLean, VA 22102
(703) 905-2000

Attachment A

BOEHRINGER MANNHEIM

The DIG System User's Guide for Filter Hybridization

Our preparations are exclusively intended for analytical purposes or for studies based on animal experiments. They must not be used for human beings since they were neither tested nor intended for such utilization.

Unsere Präparate sind ausschließlich für analytische Zwecke oder tierexperimentelle Studien bestimmt. Sie dürfen am Menschen nicht angewandt werden, weil sie hierfür weder geprüft noch vorgesehen sind.

Nuestros preparados están destinados exclusivamente a fines analíticos o estudios experimentales con animales. No deben ser administrados o aplicados a seres humanos por no estar previstos a tal efecto y no haber sido sometidos a la verificación correspondiente.

Nos préparations sont exclusivement réservées soit à des fins analytiques, soit à des études basant sur des expériences avec des animaux. Elle ne doivent en aucun cas être utilisées sur l'être humain car elles ne sont ni vérifiées, ni prévues à ces fins.

Editorial Management: Rob van Miltenburg
Barbara Rüger, Ph. D.
Stefanie Grünewald-Janho
Michael Leons
Charles Schröder

Art Direction and Typesetting: typoPlus Föll + Schulz GmbH, Mannheim

Production: Doris Eisel

ISBN 3-88630-200-8

Chapter 7 • General Considerations for Hybridization

Please review this section of general hybridization considerations before proceeding with the DIG-system. Several points are critical for successful use of the DIG-system, especially when performing chemiluminescent detection. For general information on nucleic acid hybridization, see

▶ Sambrook, J., Fritsch, E. M. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Membrane Selection

For best results, use Boehringer Mannheim's Nylon Membranes, positively charged (Cat. Nos. 1209299, 1209272, 1417240) for the transfer. This membrane has an optimal charge density, allowing it to bind the nucleic acid tightly without producing background. The nylon membranes are also specifically tested with the DIG-system to ensure optimal signal-to-noise ratios.

Other, uncharged membranes can also be used with the DIG-system. Their binding capacity is lower and therefore a lower maximum sensitivity can be achieved. In general, lower background can be expected when using uncharged nylon membranes. They are, however, not tested in combination with the DIG-System.

Nitrocellulose membranes cannot be recommended in combination with the DIG-System. They can only be used when colorimetric detection will be performed and no stripping and reprobing is planned.

Probe Concentration

In the following chapters we give recommendations for probe concentrations in the different applications. These recommendation refer to newly sythesized, DIG-labeled probe. In the package inserts of the respective labeling kits and in the „Labeling"-section of this guide, an expected yield for labeling under standard conditions is given. This must however be confirmed by estimating the yield of a labeling reaction, as is described in Chapter 5, page 33.

The recommended probe concentration must be regarded as a starting point for your hybridization. For the most accurate determination of optimal probe concentration we recommend to perform a mock hybridization (described below).

Note: If chemiluminescent detection is performed, a too high probe concentration will often lead to background. Therefore the probe concentration should not be increased above the recommended concentrations. When the chemiluminescent substrate CDP-Star™ is used, you will generally need lower probe concentrations than with chemiluminescent detection with CSPD®.

Optimization of the probe concentration – the "mock" hybridization

To prevent background problems as a result of a too high probe concentration, we recommend to optimize the probe concentration in a mock hybridization, before the actual hybridization is performed.

The mock hybridization is carried out by incubating small membrane pieces (without DNA transferred to it) with different probe concentrations in the hybridization solution and subsequent detection with the procedure of choice.

▼

For example

Probe type	Concentration in the hybridization solution		
DNA/RNA probes	1 µl*/ml	3 µl*/ml	5 µl*/ml
End-labeled oligonucleotide	1 pmol/ml	3 pmol/ml	10 pmol/ml
Tailed oligonucleotide	0.1 pmol/ml	0.5 pmol/ml	2 pmol/ml

* from the labeling reaction

The highest probe concentration that gives an acceptable background should be used for the hybridization experiment (see figure 7, 25 ng/ml).

Probe Filtration

Small particles that are contaminating the probe can be filtered out through a 0.45 µm filter. This can be performed best after addition of the probe to prewarmed (to hybridization temperature) DIG Easy Hyb and filtration of this entire hybridization solution (for information on hybridization buffers, see below). The filtration results in lower spot-like background (see figure 7). ***Note: This can only be performed when DIG Easy Hyb is used as hybridization buffer. Other hybridization buffers have components (e.g. Blocking Reagent) that cannot be filtered through a 0.45 µm filter. When you want to use another hybridization buffer and want to purify the probe, we recommend to use the procedure with High Pure PCR Product Purification Kit, described on page 40.***

Hybridization and Washing Conditions

We have found that DIG-labeled probes demonstrate the same hybridization kinetics as radiolabeled probes. Hybridization and washing conditions for DIG-labeled probes do not differ substantially from those of radiolabeled probes. The optimal hybridization and wash conditions for each probe must be determined experimentally. In this User's Guide, we provide recommendations for hybridization and washing conditions. Use the conditions given as a starting point. It may then be necessary to optimize conditions to obtain maximum sensitivity with your probe.

Labeled probes can hybridize non-specifically to sequences that bear homology but are not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matched hybrids. They can be dissociated by performing washes of various stringency. The stringency of washes can be manipulated by varying the salt concentration and temperature. For some applications, the stringency of the washes should be higher. However we recommend that you hybridize stringently (i.e., optimize hybridization conditions) rather than wash stringently.

Prehybridization/Hybridization solutions

Several hybridization buffers can be used with the DIG-System. In our experience, optimal results have been obtained with the buffers, listed page 44. The main difference with hybridization buffers described elsewhere, is the presence of Blocking Reagent. The protein in Blocking Reagent reduces the non-specific binding of probe to the membrane filter.

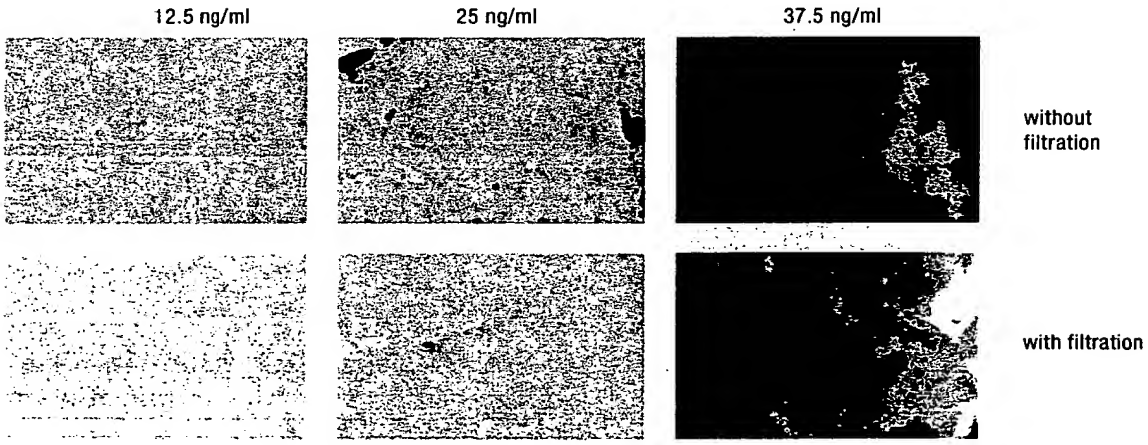


Figure 7: Mock hybridization and effect of probe filtration. Naked pieces of membrane were incubated with the indicated amounts of DIG-labeled DNA probe, with and without filtration through 0.45 µm filters, and detected with chemiluminescence.

DIG Easy Hyb*	Standard buffer	Standard buffer + 50% formamide	High SDS buffer (Church buffer)
Cat. No. 1603 558 500 ml, ready-to-use solution	5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% Blocking Reagent	50% formamide, deionized, 5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02 (w/v) SDS, 2% Blocking Reagent	7% SDS, 50% formamide, deionized, 5 x SSC, 2% Blocking Reagent, 50 mM sodium phosphate, pH 7.0, 0.1% (w/v) N-lauroylsarcosine

▲ **Table 6: Composition of recommended hybridization solutions**

*DIG Easy Hyb is a ready-to-use hybridization buffer. It is a non-toxic solution that can be used like a formamide-based hybridization buffer and is highly recommended for all membrane based applications.

Hybridization containers

You may use sealable containers or heat sealable plastic bags for the hybridization. The prehybridization and washings are generally performed in sample volume; here the membrane must be allowed to float freely, e.g. in a clean tray.

Roller tubes in combination with a hybridization oven may also be used. Use at least 6 ml of (pre-)hybridization solution per tube.

Note: The hybridization temperature should be monitored inside the roller tube. There might be a difference in the adjusted temperature and the temperature inside the bottle. Check the temperature by filling a tube with water and placing a thermometer inside the tube.

Storage and Reuse of Hybridization Solutions

One of the advantages of the DIG-System is the stability of the labeled probe. After hybridization against the blotted target, the hybridization solution still contains large amounts of unannealed DIG-labeled probe. Simply pour the solution into a plastic tube and store at –20°C for DNA probes and –70°C for RNA probes. DIG labeled probes are stable for at least 1 year when stored in this manner.

For reuse, thaw and denature by heating to +95°C for 10 min. If the hybridization solution contains formamide or if DIG Easy Hyb was used, denature at 68°C for 10 min.

Stripping and reprobing

With the DIG-System, membranes can be stripped and reprobed. To do this refer to the procedures, described on page 66.
Note: When reprobing is planned, membranes must be kept wet at all stages, after the first probe has been applied.

What to do next

At this time, proceed to the appropriate application in the "Hybridization" section of the User's Guide.

Chapter 8 • Hybridization Techniques

Southern Blotting

The DIG-System can detect 0.03 pg (chemiluminescent detection) or 0.1 pg (colorimetric detection) homologous DNA in a Southern blot format on a nylon membrane. This corresponds to the detection of a single-copy gene in < 1 µg of human genomic DNA. The procedures described here, are used routinely in our labs and have been found to give optimal results in Southern blotting, particularly in genomic Southern blotting.

Required solutions

Solutions required for Southern blotting are listed below. Refer to Appendix B for details on preparing these required solutions.

Required solution	Description
HCl	250 mM HCl
H_2O	Sterile, distilled water
Denaturation solution 1	0.5 N NaOH, 1.5 m NaCl
Neutralization solution 1	0.5 M Tris-HCl, pH 7.5; 3 M NaCl
20 x SSC buffer	3 M NaCl, 300 mM sodium citrate, pH 7.0
5 x SSC buffer	750 mM NaCl, 75 mM sodium citrate, pH 7.0
Prehybridization solution	Prepare one of the following (see Table 7 for composition and Appendix B for details on preparation) • DIG Easy Hyb • Standard buffer • Standard buffer + 50% formamide • High SDS buffer
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS

Gel Electrophoresis

Restriction digest the DNA. Prepare an agarose gel of appropriate percentage, using a high-purity, nucleic acid grade agarose, such as Agarose MP or Agarose LE (available from Boehringer Mannheim), and Tris-Borate-EDTA (TBE)- or Tris-Acetate-EDTA (TAE)-buffer. Run the digest on the gel. If desired, the gel may be stained with ethidium bromide to visualize DNA fragments and to confirm the subsequent transfer to the membrane.

Southern Transfer

The transfer of DNA from the gel to the membrane can be accomplished by one of a number of common procedures; however the following procedures are routinely used in our lab and provide optimal detection sensitivity.

Depurination (optional)

Controlled acid treatment depurinates DNA. In the subsequent alkaline denaturation of the DNA, the DNA-strand breaks at the depurinated sites, resulting in smaller, easier to transfer fragments. Depurination is an optional treatment, usually performed when fragments >10 kb must be transferred. If you are transferring small DNAs (<10 kb) or detecting only the smaller fragments in a genomic digest, it may not be necessary to depurinate the DNA. Avoid excessive acid treatment; the fragments will be too small, which results in poor detection sensitivity.

Procedure

❶ Submerge the gel in 250 mM HCl for 10 min, with shaking, at room temperature. Do not exceed 10 min.

❷ Rinse the gel with H_2O before proceeding to the "Denaturation section".

Denaturation, neutralization, and blotting

1. Submerge the gel in denaturation solution for 2 x 15 min at room temperature. Shake gently. *This treatment denatures the DNA, making it single-stranded and accessible for the later applied probe.*
2. Rinse the gel with H_2O.
3. Submerge the gel in neutralization solution for 2 x 15 min at room temperature.
4. Prepare membrane filters for Southern transfer, according to the manufacturer's recommendations. Boehringer Mannheim Nylon Membranes can be used without any prior treatments. Always use unpowdered rubber gloves when handling membranes, and manipulate the membranes with forceps at the edges only.
5. Check pH. This is especially necessary when working with nitrocellulose. The pH should be <9, but nylon membranes also tolerate a higher pH.
6. Blot the DNA from the gel by capillary transfer to the membrane, using 20x SSC buffer. Blot overnight to ensure efficient transfer of the DNA. Alternatively, the DNA can be vacuum-blotted onto the membrane; vacuum blotting can be accomplished in 1–2 h, according to the manufacturer's recommendations. Our experience indicates that capillary transfer is more efficient than vacuum transfer.

Fixation

Crosslink the DNA to the membrane by any of the following procedures.

- ▶ UV-crosslink the wet membrane without prior washing. After the UV crosslinking, rinse the membrane briefly in H_2O and allow to air-dry. *For UV-crosslinking of membranes special devices are available, that perform better than transilluminators.*

or

- ▶ Bake the membrane (Boehringer Mannheim Nylon Membranes) at +120°C for 30 min or according to the manufacturer's instructions.
- ▶ Nitrocellulose membranes must be baked at +80°C and under vacuum, to prevent spontaneous combustion of the nitrocellulose.

The membrane can now be used immediately for prehybridization, or can be stored dry at +4°C for future use.

Denaturation, neutralization, and blotting

1. Submerge the agarose gel in denaturation solution twice for 15 min at room temperature. Shake gently. This incubation denatures the DNA target prior to transfer.
2. Rinse the gel with H_2O.
3. Submerge the gel in neutralization solution twice for 15 min at room temperature to neutralize the gel.
4. Prepare membrane filters for Southern transfer according to the manufacturer's recommendations. ***Note: Always use unpowdered rubber gloves when handling membranes, and manipulate the membrane with forceps on the membrane's edges.***
5. Especially when DNA transfer to nitrocellulose membranes is intended, it is important to check the actual pH of the gel after neutralization. It should be below pH 9 (nylon membranes will tolerate a higher pH) otherwise membranes will turn yellow and break during hybridization. To check the pH of the gel, lift one edge of the gel where no DNA has been loaded, press a pH stick into it and read the pH.
6. Blot the DNA from the gel by capillary transfer to the membrane, using 20 x SSC buffer. Blot overnight to ensure efficient transfer of the DNA. Alternatively, the DNA can be vacuum-blotted onto the membrane; vacuum-blotting can be accomplished in 1–2 h, according to the manufacturer's recommendations. Our experience indicates that capillary blotting is more efficient at transferring DNA than vacuum blotting.

DNA fixation

DNA can be efficiently bound to the nylon membrane by one of the following procedures.

Procedure

1. UV-crosslink the wet membrane without prior washing. After the UV-crosslinking, rinse the membrane briefly in H_2O and allow to air-dry.
2. Alternatively, the DNA can be fixed to the membrane by baking. Bake in an oven at +120°C for 30 min (Boehringer Mannheim Nylon Membranes).
3. Nitrocellulose membranes must be baked at 80°C under vacuum to prevent spontaneous combustion of the nitrocellulose.

The membrane can now be used immediately for prehybridization, or can be stored dry at +4° C for future use.

Prehybridization and Hybridization

Prehybridization prepares the membrane for probe hybridization by blocking nonspecific nucleic acid-binding sites on the membrane. This ultimately serves to lower background. Many different prehybridization solutions have been described in the literature. However, the prehybridization solutions described here combine efficient blocking with ease of use.

As with any probe, optimal hybridization conditions for DIG-labeled probes must be determined experimentally. We strongly recommend that the time be taken to optimize each DIG-labeled probe (see the mock hybridization on page 42). The time taken for optimization will result in cleaner results and, ultimately, time savings, especially if a probe will be reused many times.

Procedure

❶ Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm^2 of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for 2 h. Longer prehybridization times are acceptable. Several membranes can be processed in the same sealed bag as long as there is sufficient prehybridization solution to cover all the membranes, and the membranes can move freely in the bag.

The optimal hybridization temperature for a specific probe will depend on the length of the probe and on the extent of sequence homology with the target sequence; therefore, it must be determined empirically. See Table 7 for recommended temperatures for different types of probes and different hybridization solutions.

▼

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
DNA	5–25 ng/ml**	DIG Easy Hyb	Hybridize overnight at 37–42° C
		Standard buffer	Hybridize overnight at 65–68° C
		Standard buffer + 50% formamide	Hybridize overnight at 37–42° C
		High SDS buffer	Hybridize overnight at 37–42° C
RNA	100 ng/ml**	DIG Easy Hyb	Hybridize overnight at 50° C
		Standard buffer + 50% formamide	Hybridize overnight at 50° C
Oligonucleotides		DIG Easy Hyb	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. Sum up 4° C for each G or C and 2° C for each A or T. Perform prehybridization and hybridization at 10° C below the obtained T_m. Hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) (in the prehybridization and hybridization solution) to prevent nonspecific hybridization signals. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
tailed	0.1–2 pmol/ml	Standard buffer	
end-labeled	1–10 pmol/ml		

▲

Table 7: Optimal hybridization conditions for different probe types.

*The conditions given here are stringent conditions applicable if probe and target have 100% homology and a GC content of about 50%.

**When CDP-Star is used for detection the recommended concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

❷ When using double-stranded DNA probes, heat in a boiling water bath for 10 min to denature the DNA. Chill directly on ice.
Single-stranded RNA probes and oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence. Prepare at least 3.5 ml hybridization solution for a blot of 10 x 10 cm.

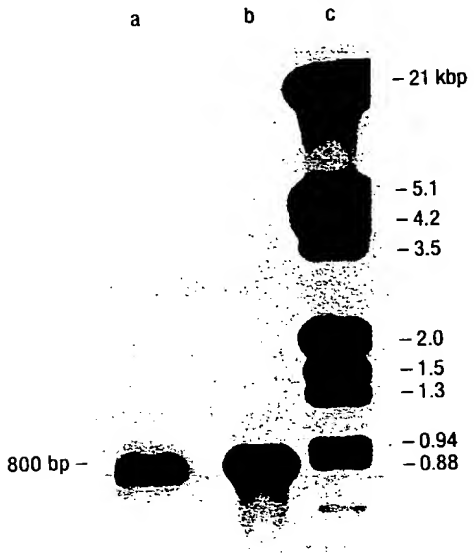


Figure 8: A typical Southern blot. Southern blot analysis of 10 µg *Hind* III-digested plant genomic DNA of transgenic tobacco ST1a containing a single copy of the *npt*-II gene (gift from M. Saul, personal communication), which was obtained by PEG-mediated direct gene transfer (M. Saul, et al., 1988). The DNA was transferred to the positively charged Nylon Membrane from Boehringer Mannheim and hybridized with a DIG-11-dUTP-labeled *Hind* III fragment of the plasmid pSHI 913 (M. Schnorf, et al., 1991) at a concentration of 25 ng DIG-labeled DNA/ml hybridization solution. The hybridization was performed in a hybridization oven in the presence of 50% formamide as described by Neuhaus-Url and Neuhaus.

A: 10 µg of *Hind* III-restricted plant DNA of ST1a releasing 1 copy of the 800 bp *npt*-II coding region.
B: 10 pg of the *Hind* III fragment of pSHI 913 reflecting 1 gene copy.
C: 40 ng of DIG-labeled Molecular Weight Marker III (Boehringer Mannheim).

Exposure time to X-ray film, to record the chemiluminescent signal was 0.5 h. The time elapsed between preincubation with the chemiluminescence substrate and exposure to X-ray film was 24 h.

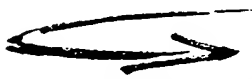
References

1. Neuhaus-Url, G. and Neuhaus, G. (1993) *Transgen. Res.* **2**, 115–120.
2. Saul, M. W., Shillito, R. D. and Negrutiu, I. (1988). In: Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. P. Verma (Eds.). Kluwer, Dordrecht, The Netherlands, pp 1–16.
3. Schnorf. M., Neuhaus-Url, G., Galli, A., Ilda, S., Potrykus, I. and Neuhaus, G. (1991) *Transgen. Res.* **1**, 23–30.

Data were kindly provided by Dr. G. Neuhaus-Url, ETH, Zurich, Switzerland.

❸ Dilute the probe in hybridization solution. See Table 7 for optimal probe concentrations.
❹ Discard the prehybridization solution from the bag. Add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 7 for selecting a hybridization solution and temperature.
❺ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (e.g., 50 ml polypropylene).
This hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Label and date the tube, and store DNA probe solutions at –20° C and RNA probe solutions at –70° C. DIG-labeled probes stored in this manner are stable for at least 1 year. For reuse, thaw and denature by heating to +95° C for 10 min. If the hybridization solution contains 50% formamide (the flash point of pure formamide is +68° C) or DIG Easy Hyb, denature at +68° C for 10 min.
❻ Wash the membrane twice, 5 min per wash, in 2 x wash solution at room temperature. These washes (steps 6 and 7) remove unbound probe, which will lead to high backgrounds if not removed.
❼ Wash the membrane twice, 15 min per wash, in 0.5 x wash solution. Long probes (>100 bp) should be washed at 68° C. For shorter probes, the wash temperature must be determined empirically.
Note: For most applications, washing in 0.5 x wash solution is stringent enough. It must be determined empirically whether it is necessary to wash with 0.1 x wash solution (0.1 x SSC, containing 0.1% SDS.

What to do next



At this time, proceed to the "Detection" division of this User's Guide, which begins on page 58.

DNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of DNA. Target DNA samples may be purified DNA, cell lysate, or PCR-amplified DNA.

The same hybridization and detection procedures used with Southern blots are also performed on DNA dot blots; therefore, proceed to the Southern prehybridization and hybridization procedures (page 47) after preparing the dot blot.

Required solutions

Products and solutions that are required for the hybridization of DNA, but not specifically required for the dot blotting procedure given here, are listed in the Southern blotting application (page 45).

Required solution	Description
DNA dilution buffer	50 µg/ml herring sperm DNA; 10 mM Tris/HCl; pH 8.0; 1 mM EDTA, pH 8.0

Procedure

1. Prepare a dilution series of your DNA target in suitable amounts.
2. Denature the DNA target in the dilutions for 10 min at +95°C, and chill immediately on ice.
3. Mark the membrane lightly with a pencil to identify each dilution before spotting. We recommend Boehringer Mannheim Nylon Membranes, positively charged.
4. Dispense 1 µl of each dilution onto the membrane. Mix dilutions well before dotting on membrane.
5. Fix the DNA to the membrane by UV crosslinking or baking in an oven at +120°C for 30 min (Boehringer Mannheim Nylon Membranes).

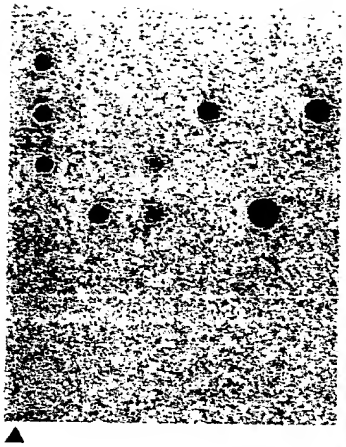


Figure 9: Chemiluminescent Dot Blot showing HLA class II typing. Human genomic DNAs from 48 patients were PCR amplified and blotted onto a nylon membrane. A HLA DRBI 01 sequence-specific oligonucleotide was 3'-end labeled with DIG-11-dUTP by Terminal transferase and hybridized to the membrane. After washing, chemiluminescent detection was performed. The blot was exposed to X-ray film for 15 min.
Data were kindly provided by Dr. A. Moine-Grenoble Transfusion, La Tronche, France.

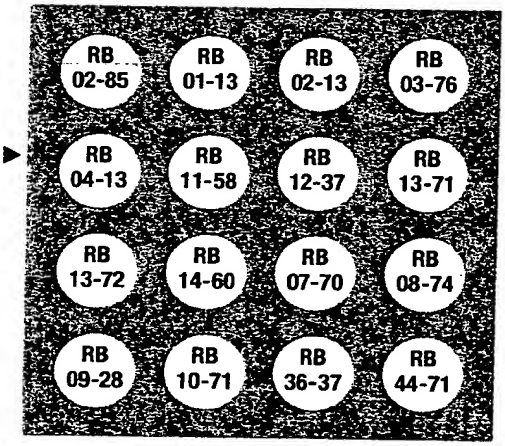
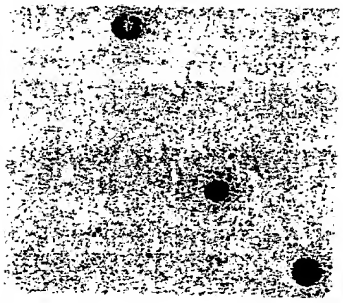


Figure 10: HLA-DR genotyping by chemiluminescent reverse Dot Blot. Sixteen sequence-specific oligonucleotides (SSOs) were blotted onto a nylon membrane. PCR-amplified target DNA (HLA-DR gene, second exons) corresponding to one individual was 3'-labeled with DIG-11-ddUTP by Terminal transferase, and hybridized to the immobilized SSOs. After washing, chemiluminescent detection was performed. The HLA-DR genotyping of this individual was found to be HLADRB1*01-DRB1*07. Data were kindly provided by Dr. J.F. Eliaou – Laboratoire d'Immunologie, Montpellier, France.



What to do next

Hybridize the samples on the dot blot according to the "Prehybridization and Hybridization" procedure described in the "Southern Blotting" application, which begins on page 45.

Colony and Plaque Hybridization

The DIG-System provides a sensitive and rapid method for detecting positive colonies or plaques in a heterologous background. Colony and plaque hybridizations have been developed to allow rapid screening of bacterial and phage recombinant libraries for specific DNA sequences. The bacterial colonies or phage particles are transferred to a nylon membrane. Alkaline treatment serves to lyse the colonies or to disassemble the phage particles. The denatured DNA is then immobilized on the membrane, followed by a proteinase K treatment to digest interfering proteins. A digoxigenin-labeled DNA, RNA or oligonucleotide probe is used for hybridization. Detection is carried out with a colorimetric or chemiluminescent immunoassay.

Recommended Membranes

We recommend to use Nylon Membranes for Colony and Plaque Hybridization, Cat. Nos. 1 699 075 (Ø 82 mm) and 1 699 083 (Ø 132 mm). The membranes are uncharged at pH 6.5 and have a pore size of 1.2 µm. The membrane discs are especially suited and tested for nonradioactive screening of phage or cosmid libraries with DIG-labeled probes and detection with highly sensitive chemiluminescent (CSPD, CDP-Star) or chromogenic substrates (NBT/BCIP, Multicolor Detection Set). The optimized retention of nucleic acids and the mechanical strength allow multiple stripping and reprobing with different probes.

Fixation of the DNA to the Nylon Membranes for Colony and Plaque Hybridization can be performed by UV crosslinking or by baking at + 80°C.

Required Solutions

Refer to Appendix B for details on preparing these additionally required solutions.

▼

Required solution	Description
Denaturation solution 1	0.5 N NaOH, 1.5 m NaCl
Neutralization solution 2	1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl
20 x SSC buffer	3 M NaCl, 300 mM sodium citrate, pH 7.0
2 x SSC buffer	0.3 mM NaCl, 30 mM sodium citrate, pH 7.0
Proteinase K	2 mg/ml Proteinase K in 2 x SSC buffer dilute Proteinase K (> 600 U/ml, 14–22 mg/ml; Cat. Nos. 1 413 783, 1 373 196, 1 373 200) 1 to 10 in 2 x SSC) *or* dissolve Proteinase K (lyophilisate, approx. 20 U/mg; Cat. Nos. 161 519, 745 723, 1 000 144, 1 092 766) 2 mg/ml in 2 x SSC
Prehybridization solution	Prepare one of the following (see Table 8 for composition and Appendix B for details on preparation) • DIG Easy Hyb • Standard buffer • Standard buffer + 50% formamide
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS

Any type of DIG-labeled DNA, RNA or oligonucleotide probe can be used for colony and plaque hybridization. However, to avoid nonspecific hybridization, use a probe that does not contain any sequences homologous to the vector. For DNA probes it is therefore recommended to use isolated inserts as template for the probe labeling.

The optimal hybridization temperature and probe concentration must be determined empirically. Table 8 offers general guidelines. ▼

Table 8: Hybridization conditions for different probe types

Probe type	Probe concentration	Hybridization solution	Time and temperature for prehybridization and hybridization*
DNA	5–25 ng/ml**	DIG Easy Hyb	Hybridize for 2 h at +42°C
		Standard buffer + 50% formamide	Hybridize overnight at +42°C
		Standard buffer	Hybridize overnight at +68°C
RNA	100 ng/ml**	DIG Easy Hyb	Hybridize for 2 h at +50°C
		Standard buffer +50% formamide	Hybridize overnight at +50°C
Oligonucleotides tailed	0.1–2 pmol/ml	DIG Easy Hyb	Hybridize for 1–6 h, hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To estimate the T_m, add 4°C for each G or C and 2°C for each A or T. Hybridize at 10°C below this estimated T_m). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) in the prehybridization and hybridization solution. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
end-labeled	1–10 pmol/ml	Standard buffer	

*The conditions given here are stringent conditions, applicable when probe and target have 100% homology and a G plus C content of about 50%.

**When CDP-Star is used for detection, the recommended probe concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

Procedure

Colony/plaque lifts

❶ Soak 2 layers of Whatman 3MM paper for each different solution: denaturation solution, neutralization solution and 2 x SSC.

❷ Pre-cool colonies or plaques on agarose plates for approx. 30 min at 4°C. For plaque lifts we recommend the use of Agarose MP (Cat. Nos. 1444964, 1388983, 1388991) for the top agar (0.7% in YT-medium).

❸ Carefully place a membrane disc onto the surface. Avoid air bubbles. Do not move the membrane once it has been applied, as transfer begins almost immediately.

❹ Leave the membrane on the plate for approx. 1 min. Mark the orientation of the membrane to the plate, in order to be able to identify the positive colonies or plaques after detection.

❺ Remove membrane disc carefully with filter tweezers and blot briefly (colonies/plaques-side up) on dry Whatman 3MM paper.

❻ Place membrane discs (colonies/plaques-side up) for 15 min (colony lifts) or 5 min (plaque lifts) on the prepared filter paper soaked with denaturation solution.

❼ Blot briefly on Whatman 3MM paper.

❽ Place membrane discs (colonies/plaques-side up) for 15 min onto the prepared filter paper soaked with neutralization solution.

❾ Blot briefly on Whatman 3MM paper.

❿ Place membrane discs for 10 min onto the prepared filter paper soaked with 2 x SSC.

⓫ Crosslink the transferred DNA with UV-light or by baking the dry membranes for at least 30 min at 80°C.

Proteinase K treatment

❶ Place membrane discs on a clean piece of aluminium foil and pipet 0.5 ml of 2 mg/ml Proteinase K on each membrane disc (0.5 ml for the discs Ø 82 mm).

❷ Distribute the solution evenly, incubate for 1 h at 37°C.

❸ Using filter paper fully wetted with dH_2O, blot membranes between the filter paper, and apply pressure by passing over the area with a ruler or a bottle.

❹ Remove cellular and agar debris by gently pulling off the upper filter paper (the debris will stick to this filter paper). Check the complete removal of all cellular debris. When necessary repeat the blotting step with a fresh piece of filter paper, soaked in water. The filters are now ready for hybridization.

Hybridization

The membranes can be hybridized in roller bottles, glass dishes, or sealed in hybridization bags. Make sure that the membranes do not stick together and are sufficiently covered with hybridization solution. We recommend no more than 4 to 5 membranes per hybridization vessel, or up to 3 membranes per roller bottle.

The volumes in the following protocol are calculated for the use of 275 ml roller bottles.

❶ Place up to 3 membrane discs (Ø 82 mm) in a roller bottle, add 60 ml prehybridization solution.
❷ Prehybridize for 1 h in a hybridization oven at the recommended temperature (see Table 8)
❸ Denature the labeled probe (double stranded probes only) by boiling for 5 min at 95–100°C. Rapidly cool on ice.
❹ Mix the denatured probe with 6 ml hybridization solution, prewarmed to hybridization temperature.
❺ Remove the prehybridization solution and add the hybridization solution.
❻ Incubate according to the recommendations in Table 8.
❼ At the end of the hybridization, pour the hybridization solution into a tube that can withstand freezing and boiling (e.g. a 50 ml polypropylene tube). *The hybridization solution can be reused several times, as long as the probe has not been depleted from solution. The DIG-labeled probes are stable for at least 1 year when stored at –20° C (DNA and oligonucleotide probes) or –70° C (RNA probes). For reuse, thaw and denature the entire mix by heating to 95° C for 10 min. When DIG Easy Hyb is used or when the hybridization solution contains formamide, denature at +68° C for 10 min.*

Stringency washes

❶ Wash the membranes twice for 5 min in ample 2 x SSC, 0.1% SDS min at room temperature with gentle agitation.
❷ Transfer the membranes to 0.5 x SSC, 0.1% SDS and wash twice for 15 min at 68° C with gentle agitation.

Note on subsequent detection

▶ Besides chemiluminescent detection or colorimetric detection with NBT/BCIP, we also recommend the use of the Multicolor Detection Set for detection. This detection method, described on page 62, allows the simultaneous detection of three different probe/target hybrids on a single filter.

What to do next

At this time, proceed to the "Detection" division of this User's Guide, which begins on page 58.

Northern Blotting

The DIG System can be readily used to detect RNA on a membrane. The results that are obtained with the DIG System are equivalent to those achieved with radioactive techniques. The same parameters in choosing hybridization conditions apply to both systems.

Probe Preparation

As is the case with radioactive probes, DIG-labeled RNA probes demonstrate stronger signals and less non-specific hybridization than DNA probes on Northern and Southern Blots. Therefore we recommend to use a RNA probe whenever possible.

If a DNA probe must be used, we recommend that you use the high SDS hybridization buffer or DIG Easy Hyb to reduce background. See Table 9 for details on hybridization solutions for different probe types.

Optimization of the Probe Concentration

Optimize the probe concentration before all hybridization experiments. This is necessary to avoid background staining, and it can be easily performed with a series of mock hybridization, where increasing concentrations of DIG-labeled probes are incubated with naked pieces of membrane or hybridized to dots of homologous DNA or RNA. This procedure is described on page 42.

Avoidance of RNase Contamination

Throughout the northern blot experiment, be careful to avoid the introduction of RNases, as RNA is susceptible to degradation even after its immobilization on a nylon membrane. We recommend sterilization of all solutions and containers that will come in contact with the RNA or northern blot. In addition to autoclaving, treat solutions and containers with DMPC (dimethylpyrocarbonate) or DEPC (diethylpyrocarbonate).

Throughout the experiment, use forceps whenever possible, and wear gloves.

Optimal Blotting Conditions

Salt concentrations between 10 x and 20 x SSC give equivalent results for the transfer of RNA from a 1% agarose formaldehyde gel to a nylon membrane. The optimal blotting duration is overnight at 4°C or room temperature.

Required solutions

Required solution	Description
Prehybridization solution	Prepare one of the following (see Table 9 for hybridization solution requirements, and see Appendix B for details on preparation) • DIG Easy Hyb • High SDS buffer • Standard buffer + 50% formamide
Hybridization solution	DIG-labeled probe, diluted in prehybridization solution
2 x Wash solution	2 x SSC, containing 0.1% SDS
0.5 x Wash solution	0.5 x SSC, containing 0.1% SDS
20 x SSC	3 M NaCl, 300 mM sodium citrate, pH 7.0; treated with DMPC
10 x SSC	1.5 mM NaCl, 150 mM sodium citrate, pH 7.0 treated with DMPC

Controls

A DIG-labeled anti-sense Actin RNA hybridization probe (Cat. No. 1498045) is available for evaluating the quality and quantity of your RNA.

Northern Transfer

1. After electrophoresis in a standard formaldehyde gel, equilibrate the gel in 20 x SSC (DMPC-treated) for 2 x 15 min.
2. Prepare a membrane filter. Wear powder-free gloves when handling the membrane, and manipulate the membrane by applying forceps to the edges.
   For best results, use Boehringer Mannheim's Nylon Membranes (Cat. Nos. 1209299, 1209272, 1417240) for the transfer. This membrane has an optimal charge density, allowing it to bind the RNA tightly without producing high backgrounds. Our nylon membrane is also specifically tested with the DIG System to ensure optimal background characteristics.
3. Blot the RNA from the gel by capillary transfer overnight at +4°C for 4 h at room temperature with 10 x or 20 x SSC (DMPC-treated).
4. UV-crosslink or bake the membrane at +120°C for 30 min.

Prehybridization and Hybridization

Before hybridization, determine the optimal probe concentration according to the mock hybridization protocol on page 42. Table 9 gives general guidelines for probe concentrations and hybridization temperatures.

❶ Place the blot in a hybridization bag containing 20 ml prehybridization solution per 100 cm² of membrane surface area. Seal the bag, and prehybridize at the anticipated hybridization temperature for at least 1 h.
Longer prehybridization times are acceptable.

Table 9: Optimal hybridization conditions for different probe types.

Probe type	Probe concentration	Hybridization solution	Temperature for prehybridization and hybridization*
RNA	50–100 ng/ml**	DIG Easy Hyb	Hybridize overnight at 68° C
		Standard buffer + 50% formamide	Hybridize overnight at 68° C
DNA	25 ng/ml**	DIG Easy Hyb	Hybridize overnight at +50° C
		High SDS buffer	Hybridize overnight at +50° C
Oligonucleotides			
tailed	0.1–2 pmol/ml	DIG Easy Hyb	Hybridize for 1–6 h; hybridization temperature varies considerably and can be approximated by considering probe length and G plus C content. (To determine the T_m, add 4° C for each G or C and 2° C for each T or A. Perform prehybridization and hybridization 10° C below the calculated T_m). To prevent non-specific hybridization signals, hybridization with a tailed oligonucleotide should be performed with 0.1 mg/ml Poly (A) in the prehybridization and hybridization solution. Additionally, 5 µg/ml of Poly (dA) may be added for further blocking.
end-labeled	1–10 pmol/ml	High SDS buffer	

*The conditions given here are stringent conditions, applicable if probe and target have 100% homology and a G plus C content of about 50%.

**When CDP-Star is used for detection, the recommended concentrations are 10–20 ng/ml DIG-labeled DNA or 20–50 ng/ml DIG-labeled RNA. Higher concentrations may cause background.

❷ Heat-denature the probe in a boiling water bath for 10 min.
Oligonucleotide probes do not require denaturation prior to dilution unless extensive secondary structure is predicted from the sequence.

❸ Dilute the probe in prehybridization solution. See Table 9 for recommended probe concentrations.

❹ Discard the prehybridization solution from the bag, and add the hybridization solution containing the DIG-labeled probe. Allow the probe to hybridize. See Table 9 for recommended hybridization conditions.

❺ At the end of the hybridization, pour the hybridization solution from the bag into a tube (with cap) that can withstand freezing and boiling (e.g., a 50 ml polypropylene tube).
This used hybridization solution contains unannealed DIG-labeled probe. The entire solution can be reused in future hybridization experiments. Store DIG-labeled DNA probes at –20°C; store DIG-labeled RNA probes at –70° C. DIG-labeled probes stored in this manner are stable for at least one year. For reuse, thaw and denature by heating to +68° C for 10 min.

❻ Wash the membrane twice, 15 min per wash, in 2 x wash solution at room temperature. These washes (steps 6 and 7) remove unbound probe, which would otherwise lead to high background.

❼ Wash the membrane twice, 15 min per wash, in 0.5 x wash solution. Wash long probes (> 100 bp) at +68° C. For shorter probes, the washing temperature must be determined empirically.

Note: The stringency of this final wash must be determined empirically. Depending on length and homology of the probe it will be necessary to adjust the salt concentration. Fully homologous probes will often require 0.1 x wash solution.

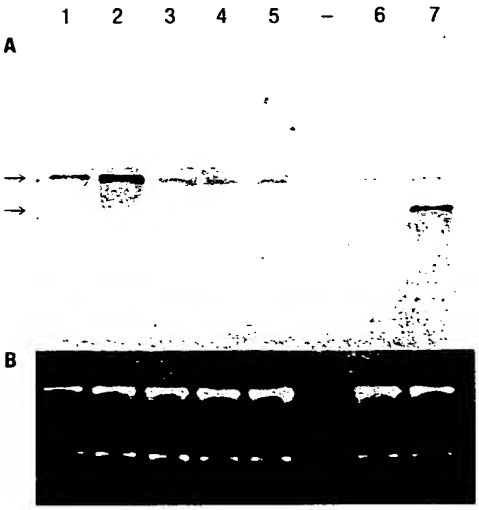


Figure 11: Example of a northern blot with a DIG-labeled RNA probe. Approximately 200 ng of total RNA from rat spinal cord (1), cortex (2), spleen (3), kidney (4), and liver (5, 6, 7) were run on a 1.5% agarose/formaldehyde gel and transferred to a nylon membrane. Specific mRNA was detected with a 2.5 kb digoxigenin-labeled antisense RNA probe derived from zinc finger cDNA. For quantification, lanes 6 and 7 contain 0.1 pg and 1 pg, respectively, of a synthetic sense RNA derived from the same cDNA.

A. 45 min exposure of the membrane 2 h after the start of the detection reaction with the chemiluminescence substrate. A 4.8 kb mRNA is detected in all tissues. The amount of mRNA in liver approximately corresponds to the 0.1 standard in lane 6 running at 2.5 kb. Arrows indicate the positions of the 18S and 28S ribosomal RNAs.

B. Photograph of the Ethidium bromide-stained 18S and 28S RNAs after transfer to a nylon membrane.

Data were kindly provided by U. Pott, Brain Research Institute, Zurich, Switzerland.

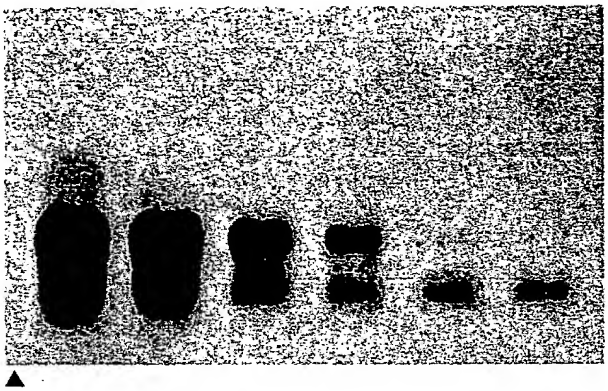


Figure 12: Comparison of extraction efficiency. RNA was extracted from different amounts of mouse heart tissue after the addition of 4 pg recovery standard.

Lane 1: Total RNA was extracted from 80 mg heart tissue by the acid guanidinium thiocyanate-phenol-chloroform method as described in reference 1. **Lanes 2 to 6:** mRNA was extracted as described in reference 2 from 80 mg (lane 2), 16 mg (lane 3), 8 mg (lane 4), 1.6 mg (lane 5), and 0.8 mg (lane 6) heart tissue. 0.8 mg of heart tissue contains only about 50 ng Poly(A)$^+$ RNA and less than 300 fg nerve growth factor (NGF) mRNA, which is below the detection limit of conventional northern blots. The RNA was glyoxylated, separated in 1.2% agarose gel, and transferred to a positively charged Nylon Membrane (Boehringer Mannheim). After hybridization with a digoxigenin-labeled cRNA probe, NGF mRNA was visualized by chemiluminescent detection. Hybridization and detection were performed under standard conditions described in DIG-labeling and detection protocols from Boehringer Mannheim.

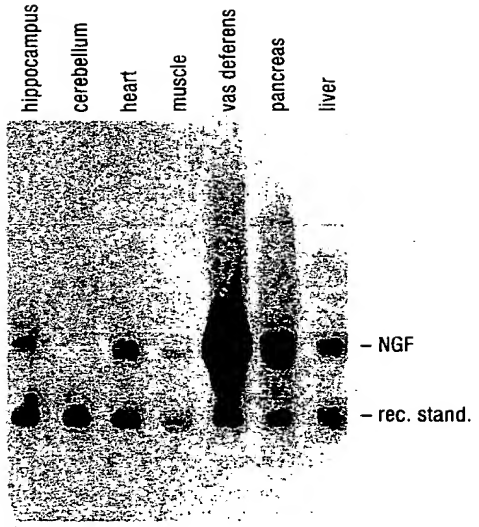


Figure 13: Extraction of mRNa from various rat tissues. mRNA was extracted from the indicated rat tissues (50 mg wet weight each) by the method described in reference 2. In order to determine the extraction efficiency, 8 pg of a shortened polyadenylated NGF recovery standard (reference 3) was added to each sample prior to mRNA extraction. Hybridization and detection were performed as described in Figure 12.

Data from Figures 12 and 13 were kindly provided by Dr. B. Hengerer, CIBA GEIGY AG, Basel, Switzerland.

References

1. Chomcynski, P. and Sacchi, N. (1987) Single-step method for RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156.
2. Hengerer, B. (1993) A rapid procedure for mRNA extraction from a large number of samples. *BioTechniques* **14**(4), 522–524.
3. Heuman, R. and Thoenen, H. (1986) Comparison between the time course of changes in nerve growth factor NGF protein levels and those of its messenger RNA, in the cultured rat iris. *J. Biol. Chem.* **261**, 9246.

What to do next

Proceed to the "Detection" division of this User's Guide, which begins on page 58.

RNA Dot Blotting

Dot blots and slot blots are rapid methods for the qualitative screening of RNA. The same hybridization and detection procedures used with Northern blots are also performed on RNA dot blots; therefore, proceed to the Northern blotting application (page 53) after completing this dot blotting procedure.

Required solutions

Solutions required for the hybridization and detection of RNA dot blots, but not specifically required by the dot blotting procedure given here, are listed in the Northern blotting application (page 53).

Required solution	Description
DMPC-treated H_2O	Sterile, distilled water, DMPC-treated with 0.1% dimethylpyrocarbonate (see page 84)
RNA dilution buffer	Mix DMPC-treated H_2O : 20 x SSC : Formaldehyde (5 : 3 : 2)

Procedure

1. Dilute the RNA sample in RNA dilution buffer.
2. Mark the membrane lightly with a pencil to identify each dilution before spotting.
3. Using a micropipettor, spot 1 µl of the RNA sample onto a dry nylon membrane. Alternatively, the sample can be applied using a slot- or dot-blotting manifold.
4. Fix the RNA to the membrane by UV crosslinking or baking in an oven at +120° C for 30 min. With nitrocellulose membranes, use a vacuum oven at +80° C for 2 h.

What to do next

Hybridize the samples on the dot blot according to the recommendations described in the nouthern blotting application, which begins on page 53.

Attachment B

JOURNAL OF BACTERIOLOGY, July 1986, p. 312–318
0021-9193/86/070312-07$02.00/0

Vol. 167, No. 1

Molecular Cloning, DNA Sequencing, and Enzymatic Analyses of Two *Escherichia coli* Pyruvate Oxidase Mutants Defective in Activation by Lipids

YING-YING CHANG* AND JOHN E. CRONAN, JR.

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 5 March 1986/Accepted 23 April 1986

Two *Escherichia coli* pyruvate oxidase (EC 1.2.2.2) mutant genes, *poxB3* and *poxB4*, were cloned on plasmid pBR322. The *poxB3* mutant oxidase which was described previously (Y. Y. Chang and J. E. Cronan, Jr., Proc. Natl. Acad. Sci. USA 81:4348–4352, 1984) was deficient in lipid activation but retained full catalytic activity. The *poxB3* mutation was located in the C-terminal half of the gene, and the nucleotide alteration has been determined by DNA sequencing of this part of the gene and by comparing the sequence with that of the wild-type strain (C. Grabau and J. E. Cronan, Jr., submitted for publication). The *poxB3* oxidase mutation is the substitution of a serine residue for Pro-536. *poxB4*, another pyruvate oxidase mutant gene, was also deficient in lipid activation. The major difference between the *poxB3* and *poxB4* oxidase was in the binding of Triton detergents. The *poxB4* mutation was also located in the C-terminal half of the gene, and sequence analysis has shown that only one nucleotide base was altered, which resulted in Ala-467 being converted to a threonine residue. The results of the amino acid substitutions in the mutant proteins, leading to the functional alteration of the enzyme, are discussed.

Escherichia coli pyruvate oxidase (pyruvate:ubiquinone-8 oxidoreductase; EC 1.2.2.2) (16, 23) is an unusually good system for studying lipid activation of an enzyme. The source of the enzyme, *E. coli*, greatly facilitates genetic and physiological analyses, and the purified enzyme has been thoroughly characterized. Pyruvate oxidase is a peripheral membrane flavoprotein that catalyzes the oxidative decarboxylation of pyruvate to yield acetate, CO_2, and reduced flavin adenine dinucleotide. Thiamine PP_i (TPP), which requires a divalent cation for binding to the enzyme, is an essential cofactor. The oxidase, a homotetramer with 62,000-M_r subunits, displays two unusual activation phenomena (16, 28). The physiologically relevant activation is by phospholipids and results in a 20-fold increase in the maximum velocity of the reaction and a 10-fold decrease in the K_m for pyruvate (16). Lipid activation can be mimicked by a limited proteolysis of the enzyme that results in cleavage of a 3,000-M_r peptide (the α-peptide) from the COOH terminus of the enzyme (26, 27). The two activation processes are closely related. Both processes require the conformational change that accompanies flavin reduction. Lipid protects the enzyme from the activating proteolytic clip (28), and the clipped enzyme lacks lipid-binding activity (28).

By isolation of mutants deficient in lipid activation, we recently demonstrated (6) that lipid activation and binding by the oxidase plays an essential role in the function of the enzyme in vivo. Subsequently, Grabau and Cronan cloned the DNA encoding the wild-type oxidase (17) and deduced the amino acid sequence of the protein from the DNA sequence of the gene (C. Grabau and J. E. Cronan, Jr., submitted for publication). We now report the amino acid alteration of the *poxB3* mutant which was previously characterized and a detailed enzymatic characterization and the sequence analysis of a second mutant gene, *poxB4*.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strains YYC198 (*poxB3*) and YYC293 (*poxB4*) are isogenic derivatives of *E. coli* K-12 that carry a deletion of the *aceEF* (pyruvate dehydrogenase) genes. Strain YYC198 is a nonlysogenic derivative of strain YYC164 *poxB3* (5). Strain YYC293 (*poxB4*) is another pyruvate oxidase mutant isolated independently from YYC63 (5). Strain CG3 is a *recA* nonlysogenic derivative of strain YYC162 (5, 17) (Δ*aceEF pfl-1 pps-4 poxB1*). Strain CY379 is a Δ*aceEF leu* derivative of strain UB1005 (17). Strain LE392 is an *hsdR* derivative of *E. coli* K-12. Strain JM103 and bacteriophage M13mp11w were obtained from J. Messing (24).

The media used were described previously (4). Restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, *E. coli* DNA polymerase I (large fragment), *Xho*I linker (5'CCTCGAGG3'), and 15-mer oligonucleotide primer were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; International Biotechnologies, Inc., New Haven, Conn.; and New England BioLabs, Inc., Beverly, Mass. [^{32}P]dATP was obtained from Amersham Corp., Arlington Heights, Ill. Synthetic phospholipids were obtained from Calbiochem-Behring, La Jolla, Calif. Ribo- and deoxyribonucleotides, alkyl sulfates, antibiotics, Triton X-100, Triton X-114, and other chemicals were from Sigma Chemical Co., St. Louis, Mo.

Preparation of plasmids, DNA sequencing, and other methods involving DNA. Purified plasmids were isolated and prepared by the mild Triton X-100-lysozyme lysis of chloramphenicol-amplified cultures (22) followed by two cycles of cesium chloride-ethidium bromide gradient centrifugation (21). For strains which are lysed poorly by this method (e.g., CG3), the large-scale alkaline lysis method (21) was used. The gradient centrifugation was modified (O. M. Griffith, Beckman Instruments, Inc., Palo Alto, Calif., personal

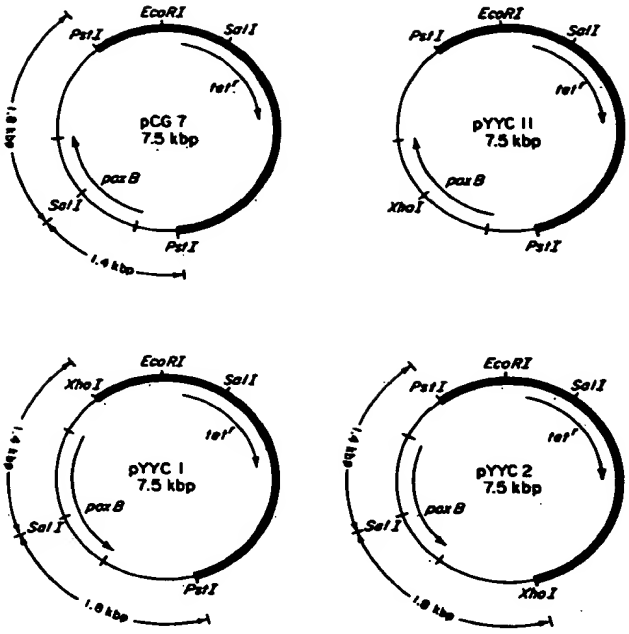


FIG. 1. Maps of plasmids used. The thick lines are pBR322 sequences; *tet^r* and *poxB* are the DNA regions encoding tetracycline resistance and pyruvate oxidase, respectively. The arrows indicate the direction of transcription.

communication) by centrifugation at 55,000 rpm for 16 h followed by centrifugation for 45 min at 40,000 rpm with a Beckman Ti 70.1 rotor. For rapid minipreparation of plasmids, sodium dodecyl sulfate (SDS)-alkaline lysis was used (21).

The conditions used for restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were those recommended by Maniatis et al. (21) or the suppliers. DNA sequencing was done by the dideoxynucleotide chain-termination method of Sanger et al. (29). DNA fragments to be sequenced were cloned into the multiple cloning site of phage M13mp11w. The methods for cloning and selection of recombinant phages were those of Messing (24). DNA fragments from restriction enzyme digestions were analyzed by electrophoresis on a 1% agarose gel in Tris borate buffer (21) and visualized by ethidium bromide staining. Extraction of the DNA bands from the gel by using either DEAE-cellulose paper (DE-81; Whatman, Inc., Clifton, N.J.) or, in later experiments, a DEAE membrane (NA-45; Schleicher & Schuell, Inc., Keene, N.H.) was by the method of Dretzen et al. (14). The procedure for $CaCl_2$-mediated transformation was essentially that of Cohen et al. (11).

Construction of plasmid pYYC11. Plasmid pYYC11 (Fig. 1) was prepared from pCG10, which was kindly provided by C. Grabau of this laboratory. Plasmid pCG10 is a derivative of pACYC177 (3) and contains a 3.2-kilobase-pair (kbp) *PstI* fragment of DNA which encodes a modified *poxB* gene (a *XhoI* linker was inserted at the *SalI* site in the middle of the gene) inserted into the *PstI* site of the ampicillin resistance (Ap^r) gene of pACYC177. The construction of the plasmid and its use in the isolation of plasmids carrying *poxB3* and *poxB4* are described elsewhere (7).

Construction of plasmids pYYC1 and pYYC2. A derivative of plasmid pCG7 (Fig. 1) in which the 3.2-kbp *PstI* insert which encodes the *poxB* gene is inserted in the orientation opposite to that in pCG7 was partially digested with the restriction endonuclease *PstI*, and the DNA fragments were separated by agarose gel electrophoresis. The 7.5-kbp (full length) linearized plasmid was extracted from the gel by using DEAE-cellulose paper. This 7.5-kbp DNA consisted of a mixture of plasmids cut at one of the two *PstI* sites of the plasmid. The protruding 3′ ends of the mixture were converted to blunt ends with T4 DNA polymerase and then ligated to an *XhoI* linker, as described by Maniatis et al. (21). The resulting plasmid was transformed into strain LE392, and plasmids of several transformants were isolated and mapped with several restriction enzymes. Two plasmids, pYYC1, which has an *XhoI* site 6.8 kbp clockwise from the unique *EcoRI* site, and pYYC2, which has an *XhoI* site 3.6 kbp clockwise from the *EcoRI* site, were obtained (Fig. 1).

Synthesis and purification of 22-mer oligonucleotide primer. A 22-mer oligonucleotide (5′-GGCATGTCCTTATTATG ACGGG-3′) with a DNA sequence complementary to the DNA sequence just downstream from the termination codon of the oxidase was synthesized by the University of Illinois Genetic Engineering Facility by using an Applied Biosystems DNA synthesizer and phosphoromidite chemistry. The 22-mer was separated from the failed oligonucleotides by the binding of its trityl group to a C-18 Sep-Pak column (Waters Associates, Inc., Milford, Mass.), as described by Lo et al. (20) and Zoller and Smith (30), with some modifications. The column was washed with 10 ml of acetonitrile, with 5 ml of 30% acetonitrile in 100 mM NH_4HCO_3, and finally with 10 ml of 25 mM NH_4HCO_3. The 0.4-ml sample (ca. 0.8 mg) in 25 mM NH_4HCO_3 was applied to the column, and the column was washed with 0.6 ml of 25 mM NH_4HCO_3 and eluted sequentially with 10 ml of 10% acetonitrile in 25 mM NH_4HCO_3, 10 ml of 10% acetonitrile in H_2O, and 5 ml of 30% acetonitrile in H_2O. The failed sequences were eluted in the first fraction, whereas the 22-mer appeared mainly in the second fraction, with some tailing into the final fraction. The purity was verified by polyacrylamide gel electrophoresis on a 20% gel in the presence of 7 M urea (21).

Preparation, purification, and assay of pyruvate oxidase. Crude extracts of bacteria were prepared as previously described (4). The *poxB4* mutant oxidase was purified from strain CG3, carrying plasmid pYYC16, which overproduced the enzyme about 20-fold. Because of the greater initial specific activity, the purification scheme was modified somewhat from that used previously for the purification of the wild-type and *poxB3* mutant oxidases (6). Packed cells (ca. 120 g [wet weight]) from a 20-liter fermentor culture were used as the enzyme source, and only a single ion-exchange chromatography column (DEAE-cellulose [DE52; Whatman] rather than DEAE-Sepharose or DEAE-Sephadex) was run. The enzyme was >90% pure as judged by SDS-polyacrylamide gel electrophoresis and had a specific activity (in the presence of 20 μM SDS) of 6×10^4 U/mg of protein.

The oxidase was assayed spectrophotometrically with $Na_3Fe(CN)_6$ as the electron acceptor (4). Activation was assayed after incubating the enzyme with pyruvate, TPP, $MgCl_2$, and the activator for 30 min at room temperature. One unit of enzyme activity was 1 nmol of pyruvate decarboxylated per min.

The purified wild-type *E. coli* K-12 pyruvate oxidase (encoded by the related plasmid pCG7) was kindly provided by P. Porter and R. Gennis of this institution and had a

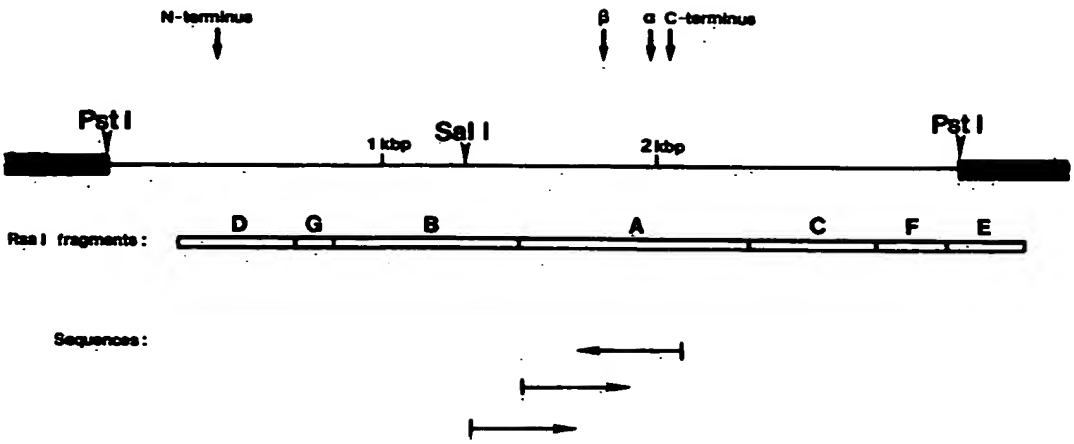


FIG. 2. DNA sequencing scheme for mutant *poxB3* and *poxB4* genes. The 3.2-kbp *PstI-PstI* insert which encodes the *poxB* gene of pCG7 is shown. α and β indicate the locations of α-chymotripsin cleavage sites resulting in activated and inactivated pyruvate oxidase, respectively. The arrows indicate the DNA regions sequenced.

specific activity in the presence of 20 μM SDS of 9×10^4 U/mg of protein.

Triton X-114 partition. A precondensed sample of Triton X-114, obtained as described by Bordier (2), was kindly provided by C. Grabau of this laboratory. Oxidase samples (20 μg) were mixed with Triton X-114 at a final concentration of 1% detergent in 10 mM Tris hydrochloride (pH 7.4) containing 150 mM NaCl. The conditions for the separation of phases were those described by Bordier (2), except that the sucrose cushion was omitted.

Electrophoresis. SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels was performed as described previously (5).

RESULTS

Molecular cloning of the *poxB3* allele. The method used, which is described in detail elsewhere (7), involved construction of a plasmid, pYYC11 (Fig. 1), in which the *SalI* site of the *poxB* gene had been converted to a unique *XhoI* site. The *XhoI* plasmid was then UV irradiated (8) and transformed into a *poxB3 recA*$^+$ strain, and plasmids of homogenotes were selected by their resistance to *XhoI* digestion and identified by the appearance of the *SalI* site originating from the chromosomal *poxB3* gene by homologous recombination. One or two cycles of *XhoI* cutting of the plasmids of pooled transformants resulted in populations of transformants which carried 50 to 100% homogenotes (7). The plasmids of individual transformants were screened for loss of the *XhoI* site and gain of the *SalI* site of the *poxB* gene. One plasmid, pYYC13, had the expected restriction map, and strains carrying the plasmid overproduced a protein with lipid-activation properties identical to those previously observed for the *poxB3* enzyme (6). It should be noted that introduction of pYYC13 into a strain carrying the completely defective *poxB1* allele resulted in white colonies which were unable to reduce a tetrazolium redox indicator in the presence of pyruvate (colonies of wild-type strains are red), although the oxidase level measured with an artificial electron acceptor exceeded the level found in extracts of a wild-type strain without a plasmid. The introduction of pYYC13 into strain CY379 also failed to allow growth on an acetate-deficient medium, whereas plasmids carrying the wild-type allele allow growth under these conditions (17). These results strongly suggest that the *poxB3* mutant is more defective in vivo than we previously reported (on the order of <1% of the normal activity).

Localization of the *poxB3* mutation on the cloned gene. Grabau and Cronan (17) have cloned the wild-type *poxB* gene on a pBR322 derivative, plasmid pCG7. These workers have also determined the entire 1.7-kbp DNA sequence encoding the oxidase (Grabau and Cronan, submitted). By using these data, we constructed two plasmids, pYYC1 and pYYC2 (Fig. 1), derived from pCG7 (17), in which one of the two *PstI* sites on each plasmid had been converted to an *XhoI* site (see Materials and Methods). To locate the *poxB3* lesion, plasmid pYYC13 (which carries *poxB3*) was digested with the restriction endonucleases *PstI* and *SalI*. The 1.8- and 1.4-kbp DNA fragments were separated by agarose gel electrophoresis and were extracted from the gel. The 1.4- and 1.8-kbp DNA fragments encode the N-terminal and C-terminal halves of the oxidase, respectively (Fig. 1). Plasmid pYYC1 was digested to completion with *PstI* and digested partially with *SalI*. The resulting 5.7-kbp DNA fragment of pYYC1 was isolated from the gel and ligated to the 1.8-kbp *PstI-SalI* DNA fragment isolated from plasmid pYYC13. The new plasmid contained a hybrid *pox*B gene, the N-terminal half of the wild-type gene, and the C-terminal half of the *poxB3* mutant gene. The reciprocal construction (the 6.1-kbp pYYC2 DNA fragment ligated to the 1.4-kbp *PstI-SalI* pYYC13 DNA fragment) was also made. Both plasmids were transformed into strain CG3, and the transformants were tested on the indicator medium. The strain carrying the plasmid which contained the COOH-terminal half of the *poxB3* mutant gene gave white colonies (oxidase negative) on tetrazolium-containing medium, whereas the strain carrying the plasmid which contained the N-terminal half of the *poxB3* gene had the wild-type oxidase-positive phenotype (red colonies). The results indicated that the *poxB3* mutation resides on the 1.8-kbp *PstI-SalI* DNA fragment of pYYC13. Because only about 0.77 kbp of this DNA fragment are *poxB* gene sequences (Fig. 2), only this segment of the *poxB3* gene DNA was sequenced to locate the mutational lesion.

DNA sequence of the *poxB3* gene fragment. The scheme for DNA sequencing is shown in Fig. 2. The 1.8-kbp *PstI-SalI*

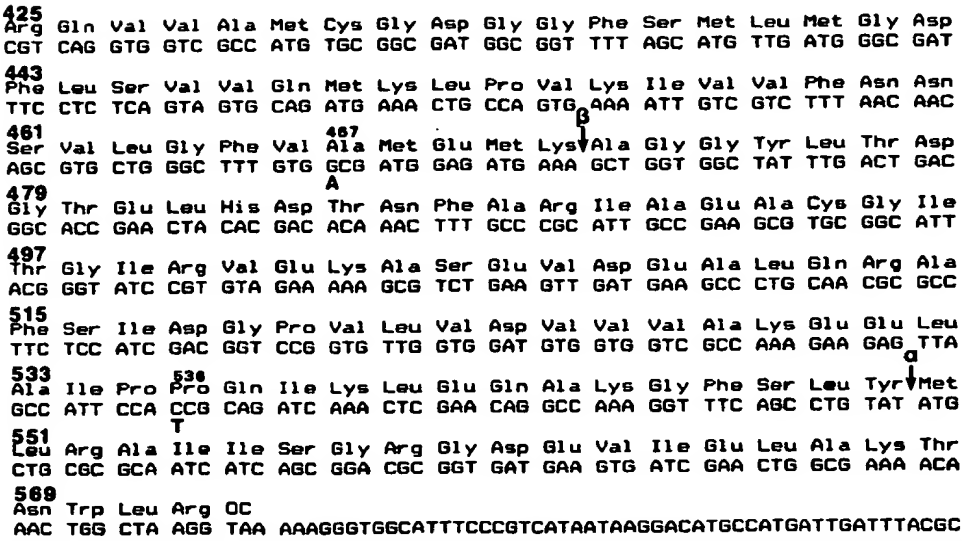


FIG. 3. DNA sequence that encodes the C-terminal region of pyruvate oxidase. The *poxB4* and *poxB3* mutations are shown at residues 467 and 536, respectively. The arrows indicate the sites of α-chymotrypsin cleavage; in the presence of substrate and cofactors, α-chymotrypsin cleavage resulted in a 3,000-M_r α-peptide (α), whereas in the absence of substrate and cofactors, α-chymotrypsin cleavage resulted in an 11,000-M_r β-peptide (β).

DNA fragment of pYYC13 and *Rsa*I fragment A of the *poxB3* gene was cloned in M13mp11w. The direction for sequencing of the 1.8-kbp *Pst*I-*Sal*I fragment was from *Sal*I toward *Pst*I, whereas the *Rsa*I A fragment had been sequenced in both directions. Either the universal 15-mer oligonucleotide or a synthetic 22-mer oligonucleotide (see Materials and Methods) was used as a primer. The DNA sequence, from the *Sal*I site to the termination codon (about 770 bases), of the *poxB3* gene had only one nucleotide base altered in comparison with the sequence of the wild-type gene. A C-to-T change found at nucleotide base 114 upstream from the termination codon resulted in the conversion of Pro-536 to a serine residue in the encoded protein (Fig. 3).

Isolation, cloning, and DNA sequencing of the *poxB4* mutant gene. Strain YYC293 (*poxB4*) was another pyruvate oxidase mutant isolated by mutagenesis with *N*,*N*-nitrosoguanidine. Strains YYC198 (*poxB3*) and YYC293 (*poxB4*) are isogenic except for the *poxB* allele but were obtained from independent experiments. The properties of the pyruvate oxidase of the *poxB4* mutant were quite similar to those of the *poxB3* oxidase. Crude extracts of the *poxB4* mutant contained negligible amounts of pyruvate oxidase activity (Table 1), but proteolysis by α-chymotrypsin in the presence of substrate and cofactor resulted in activity comparable to that of the wild-type strain. It was also found that Triton X-100 did not activate the *poxB4* oxidase when either crude or lipid-depleted extracts of the mutant were tested. In these respects, the *poxB4* enzyme was similar to the *poxB3* oxidase. However, Triton X-100 inhibited proteolytic activation of the *poxB4* oxidase (see below), whereas it did not inhibit activation of the *poxB3* oxidase. This indicated that the *poxB4* oxidase represented a second class of pyruvate oxidase mutants in which the oxidase retained some affinity for lipids (accounting for the protection from α-chymotrypsin cleavage), but in which the lipid binding failed to result in enzyme activation.

TABLE 1. Activity of wild-type and two mutant oxidases under various conditions[a]

Activator(s)	Activity of:					
	Crude extracts (U/mg of protein)			Purified oxidase (%)		
	pox$^+$	*poxB4*	*poxB3*	*pox*$^+$	*poxB4*	*poxB3*
None	105	2	17	6.8	1.4	5
SDS				100	100	100
α-Chymotrypsin	130	98	111	99	101	72
Triton X-100	223	5	17	221	8.8	20
Triton X-100 plus α-chymotrypsin		49	104		55	69

[a] The activities of the crude extracts are given in units per milligram of protein, whereas those of the purified oxidases are given relative to the activity observed in the presence of 20 μM SDS (100 %). The concentrations of the activators tested were as follows: SDS, 20 μM; α-chymotrypsin, 20 μg/ml; Triton X-100, 2.2×10^{-2} M. The data for the purified *poxB3* oxidase were taken from reference 12 to facilitate comparisons.

The molecular cloning and DNA sequencing of the *poxB4* gene followed the same route as that of the *poxB3* gene. Plasmid pYYC11 was UV-irradiated and transformed into strain YYC293 (*poxB4*), and subsequently a plasmid (pYYC16) encoding the *poxB4* gene was isolated as described for the isolation of pYYC13. Strain CG3 transformed with pYYC16 gave white colonies on the pyruvate-tetrazolium medium, indicating that even when it was overproduced, the *poxB4* mutant oxidase was not functional in vivo. By exchanging gene fragments with the wild-type gene, it was found that the *poxB4* lesion, like that of *poxB3*, was located within the C-terminal half of the oxidase gene. The sequence of the C-terminal half of the *poxB4* gene was obtained from the 1.8-kbp *Pst*I-*Sal*I and *Rsa*I A DNA fragments of pYYC16 (also see Fig. 2). Among the 770 bases sequenced, only one nucleotide base was altered, which resulted in Ala-467 being converted to a threonine residue (Fig. 3).

Purification and lipid activation of the *poxB4* mutant oxi-

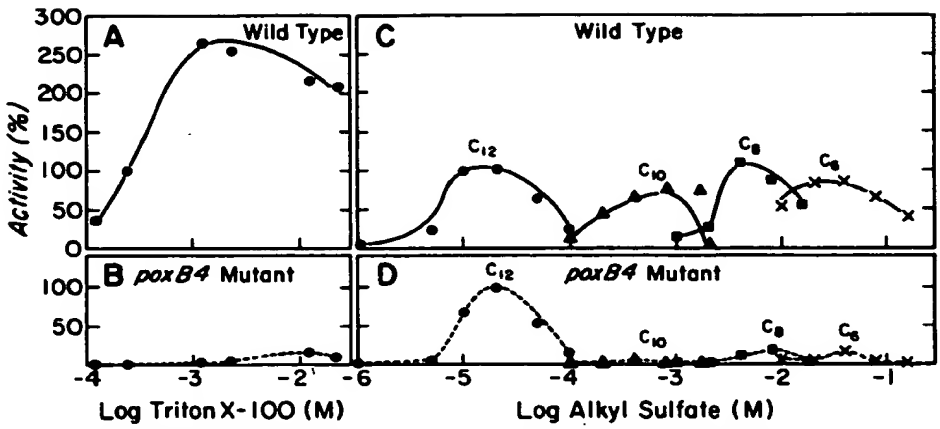


FIG. 4. Activation of purified oxidase preparations by Triton X-100 or alkyl sulfates of different chain lengths. The activities are given relative to that obtained in the presence of 20 μM SDS. Shown is activation of the wild-type (A and C) and mutant *poxB4* (B and D) oxidases by Triton X-100 (A and B) and sodium alkyl sulfates (C and D). Symbols: ●, SDS; ▲, decyl sulfate; ■, octyl sulfate; ×, hexyl sulfate. The levels of activity seen in the absence of an activator have been subtracted from the data; these values were 6.8 and 1.4% of the activities in the presence of 20 μM SDS for the wild-type and mutant oxidases, respectively.

dase. Since the *poxB4* gene had been cloned on plasmid pYYC16, the purification of the oxidase from the strain overproducing the *poxB4* mutant oxidase was relatively simple (see Materials and Methods).

The activity of the purified *poxB4* oxidase was tested with several activators (e.g., Triton X-100 and alkyl sulfates and diacyl phosphatidylcholines of various chain lengths). Like the *poxB3* oxidase, the *poxB4* oxidase was not activated by the nonionic detergent Triton X-100 (Fig. 4), although Triton X-100 is the best activator of the wild-type oxidase (1). Alkyl sulfates from hexyl sulfate (C_6) to dodecyl sulfate (C_{12}) activated the wild-type oxidase, as shown previously (1, 6), but only SDS activated the *poxB4* oxidase (Fig. 4). SDS at 20 μM activated the *poxB4* oxidase to the same level as did α-chymotrypsin. A series of chemically synthesized phosphatidylcholines with various fatty acid chain lengths from dibutyryl (C_4) to dipalmitoyl (C_{16}) were also tested. In general, the activation curves were quite similar to those of the *poxB3* oxidase described previously (6). Also, like the *poxB3* oxidase, the purified *poxB4* mutant oxidase was activated by dipalmitoyl phosphatidylethanolamine, dipalmitoyl phosphatidylglycerol, and cetyltrimethylammonium bromide. In summary, the general properties of the *poxB4* oxidase were quite similar to those of the *poxB3* oxidase. A detergent (e.g., SDS) which binds tightly to the wild-type protein activated the mutant enzyme, whereas detergents which bind only weakly to the wild-type oxidase were extremely poor activators of the mutant oxidase. The short-chain diacyl phosphatidylcholines were poor activators of the mutant oxidase, but the tight-binding longer-chain diacyl phosphatidylcholine (C_{16}) activated the mutant oxidase to a level similar to that of the wild-type oxidase.

The major enzymatic difference between the *poxB3* and *poxB4* oxidases was in the binding of Triton detergents. In crude extracts of the *poxB4* mutant, we found that Triton X-100 could decrease the extent of α-chymotrypsin activation by 50% (Table 1). A similar result was found for the purified *poxB4* oxidase (Table 1). Triton X-100 inhibited α-chymotrypsin activation of the purified *poxB4* oxidase by about 45% compared with the level of activation seen in the absence of the detergent. In contrast, α-chymotrypsin activation of the *poxB3* oxidase was unaffected by the presence of Triton X-100 (Table 1). Polyacrylamide gel electrophoresis under denaturing conditions showed (Fig. 5) that Triton X-100 also gave the *poxB4* enzyme partial protection to α-chymotrypsin cleavage. The protective effect of other lipid activators was as expected; those compounds which activated the *poxB4* oxidase protected the enzyme from the protease, whereas those compounds that failed to activate the oxidase also failed to protect the enzyme from protease clipping.

Triton binding was assayed more directly by using the partition technique of Bordier (2) to examine binding to

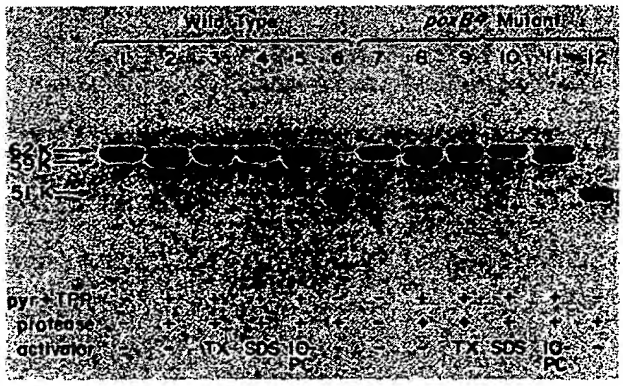


FIG. 5. Protection of the wild-type and mutant oxidases from proteolytic clipping. A 10% polyacrylamide gel run in the presence of SDS and stained with Coomassie blue is shown. Each sample (15 μl) contained purified oxidase (0.2 mg/ml), 73 mM sodium phosphate buffer (pH 6.0), 5% glycerol, and 13 mM $MgCl_2$. Added to various samples were 0.2 M pyruvate, 1.3 mM TPP, or chymotrypsin (26 μg/ml). The lipid activators added were 2.2 mM Triton X-100 (TX), 200 μM SDS, and 2 mM didecanoyl phosphatidylcholine (10-PC). After 30 min at room temperature, the sample was diluted 1:1 with sample buffer containing SDS, boiled for 2 min, and loaded onto the gel. Lanes 1 to 6 and 7 to 12 contained the wild-type and mutant oxidases, respectively. The locations of the uncleaved oxidase subunit (62K) and the proteolytically activated (59K), and nonactivated (51K) forms are shown.

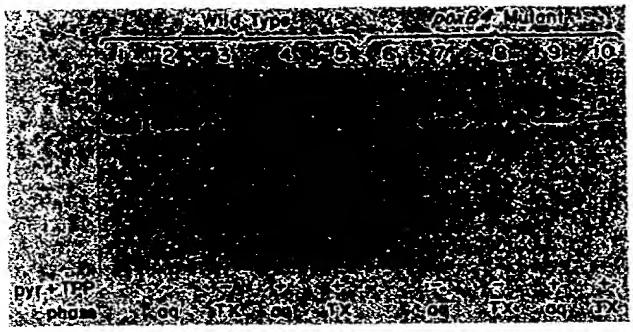


FIG. 6. Partition of the wild-type and *poxB4* oxidases into aqueous and Triton X-114 phases under activated or nonactivated conditions. A 10% polyacrylamide gel run in the presence of SDS and stained with Coomassie blue is shown. Lanes 1 and 6 contained control samples (2 μg) of the wild-type and mutant *poxB4* oxidases, respectively, without treatment. Activated samples (0.2 ml) containing the wild-type or mutant *poxB4* oxidase (20 μg), 7.5 mM Tris hydrochloride (pH 7.4), 112 mM NaCl, 1% Triton X-114, 0.1 M pyruvate, 0.1 mM TPP, and 10 mM $MgCl_2$ were mixed at 0°C and then incubated at 30°C for 3 min. Nonactivated samples lacked pyruvate and TPP-Mg^{2+}. The conditions for separation of the upper aqueous and the lower Triton phase were the same as those described by Bordier (2). After phase separation 1/10 of the samples (2 μg of oxidase) of each phase was loaded onto the gel after dilution with an equal volume of sample buffer containing SDS and boiling for 2 min. aq, Aqueous phase; TX, Triton X-114 phase.

Triton X-114, a homolog of Triton X-100 which has a slightly longer population of polyoxyethylene chains (the ranges of the chain lengths of the two detergents overlap). At 0°C, solutions of Triton X-114 are homogeneous, but at temperatures above 20°C (the cloud point), separation into two phases occurs, with the lower phase containing virtually all of the detergent. Bordier (2) showed that hydrophobic proteins partition into the lower detergent phase, whereas hydrophilic proteins are found in the upper (detergent depleted) aqueous phase. Grabau and Cronan (unpublished result) found that the wild-type oxidase completely partitioned into the detergent phase, but only in the presence of TPP-Mg^{2+} and pyruvate. In the absence of the substrate-cofactor mixture, all of the oxidase remained in the aqueous phase. The *poxB4* oxidase also required pyruvate and TPP-Mg^{2+} for partition into the detergent phase, but the extent of partition was only about half that seen with the wild-type oxidase (Fig. 6). It should be noted that, like Triton X-100, Triton X-114 is an excellent activator of the wild-type oxidase (1) but failed to activate the *poxB4* oxidase. It should also be noted that the *poxB4* enzyme reduced the water-soluble ubiquinone UQ1 and thus retained a functional ubiquinone reductase activity (18) (data not shown).

DISCUSSION

The major finding of this work was that small changes in a protein could have dramatic effects on lipid binding. Moreover, the finding that the *poxB4* mutant retained Triton-binding ability without being activated by the detergent is the first indication that lipid binding by pyruvate oxidase can be dissociated from enzyme activation by the bound lipid. Our finding that both of the mutants deficient in lipid activation have single amino acid substitutions in the C-terminal half of the protein is consistent with the suggestions of others (16, 23) that the lipid-binding site is within this segment of the protein. However, neither amino acid substitution is within the α-peptide (the peptide released upon the proteolytic clipping which results in activation and loss of lipid binding), and thus the α-peptide region constitutes only part of the lipid-binding site. The isolation of additional mutants should identify the parts of the protein involved in lipid binding.

The *poxB3* oxidase mutation is the substitution of a serine residue for Pro-536. Proline and serine residues have similar hydrophobicities (15), and Pro-536 is not located in a particularly hydrophobic region of the oxidase (Fig. 3). Therefore, we doubt that the substitution alters a surface that directly interacts with the lipid activator. We believe that it is more likely that the *poxB3* substitution hinders the conformational change triggered by the flavin reduction that exposes the lipid-binding site (16, 23). This conformational change seems quite localized because no major alterations of the overall structure of the molecule have been observed (23). The specific proteolytic clipping of the reduced oxidase which results in enzymatic activation suggests the movement of a COOH-terminal portion of the protein located near the α-peptide region. Several results indicate that regions on either side of Pro-536 are involved in lipid binding. On the C-terminal side are the α-peptide region and the Lys-544 binding site reported by Hamilton et al. (S. Hamilton, M. Recny, and L. P. Hager, personal communication), and on the N-terminal side is the putative lipid-binding region identified by the *poxB4* mutation (see below). Thus, Pro-536 and neighboring residues could provide a hinge to facilitate the movement of these and other segments of the protein in the formation of the lipid-binding site.

Proline residues have special abilities to change the direction of polypeptide chains. Three main structures are involved, β-turns (9, 10), *cis*-proline residues (9), and proline helices (9). It seems unlikely that the most abundant of these structures, β-turns, would account for the properties of the *poxB3* oxidase because serine, the amino acid substituted for Pro-536, is also very frequently found in β-turns (10). The unique ability of proline residues to isomerize to a *cis* form could account for the *poxB3* phenotype. The substituted serine lacks isomerization ability; therefore, a significant change in the geometry of the lipid-binding site could result. The possibility that proline isomerization might be involved in the lipid activation of the oxidase is suggested by the fact that both proline isomerization (19) and full lipid activation of the reduced oxidase require several minutes for completion (13). The loss of one of two adjacent proline residues in the *poxB3* oxidase raises the possibility of a proline (collagen) helix in this region. However, few globular proteins contain such helices (9).

The *poxB4* lesion results in conversion of the Ala-467 residue to a threonine residue. The Ala-467 residue lies within one of the more hydrophobic regions of the protein (Fig. 3), and the substitution should result in a significant (about 0.7 kcal [ca. 2.9 kJ]/mol [15]) decrease in the hydrophobicity of the peptide segment. The fact that the *poxB4* oxidase was equally distributed between the aqueous and Triton X-114 phases in the presence of substrate and cofactors during the partition is consistent with the decrease in hydrophobicity predicated on the amino acid substitution. Thus, this region of the protein may be part of the lipid-binding site, and its decrease hydrophobicity could account for the inability of loosely binding lipids to activate the *poxB4* enzyme (see reference 6 for details of this argument). The finding that the Triton detergents were unique in binding to the *poxB4* oxidase without activating the enzyme suggests that these detergents may interact with the oxidase in an

unusual manner. Indeed, previous workers have shown that Triton detergents give the highest level of activation of any lipid activator (1) and that only the Tritons, among the activators tested, increased the dissociation constant of the activated enzyme for TPP-Mg^{2+} (25).

The lack of a crystal structure for the oxidase precludes any definitive analysis of the mutant proteins at this time. However, it is now clear that small and rather subtle changes in this protein result in large changes in its ability to bind lipids in vitro and result in the loss of protein function in vivo. Moreover, it appears that genetic analysis will be able to provide much needed information on the mechanisms of protein-lipid interactions.

ACKNOWLEDGMENTS

We thank C. Grabau for providing the DNA sequence data for pyruvate oxidase before publication. We also thank M. Narasimhan and C. Grabau for helpful discussions concerning DNA sequencing technique.

This work was supported by research grant PCM 8308778 from the National Science Foundation.

LITERATURE CITED

1. **Blake, R., II, L. P. Hager, and R. B. Gennis.** 1978. Activation of pyruvate oxidase by monomeric and micellar amphiphiles. J. Biol. Chem. 253:1963–1971.
2. **Bordier, C.** 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256:1604–1607.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134:1141–1156.
4. **Chang, Y.-Y., and J. E. Cronan, Jr.** 1982. Mapping nonselectable genes of *Escherichia coli* by using transposon Tn*10*: location of a gene affecting pyruvate oxidase. J. Bacteriol. 151:1279–1289.
5. **Chang, Y.-Y., and J. E. Cronan, Jr.** 1983. Genetic and biochemical analyses of *Escherichia coli* strains having a mutation in the structural gene (*poxB*) for pyruvate oxidase. J. Bacteriol. 154:756–762.
6. **Chang, Y. Y., and J. E. Cronan, Jr.** 1984. An *Escherichia coli* mutant deficient in pyruvate oxidase activity due to altered phospholipid activation of the enzyme. Proc. Natl. Acad. Sci. USA 81:4348–4352.
7. **Chang, Y. Y., and J. E. Cronan, Jr.** 1985. Selection for the transfer of phenotypically non-selectable chromosomal mutations to recombinant plasmids through introduction of an altered restriction site. Gene 40:353–357.
8. **Chattoraj, D. K., K. Cordes, M. L. Berman, and A. Das.** 1984. Mutagenesis and mutation transfer induced by ultraviolet light in plasmid-cloned DNA. Gene 27:213–222.
9. **Chothia, C.** 1984. Principles that determine the structure of proteins. Annu. Rev. Biochem. 53:537–572.
10. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predications of protein conformation. Annu. Rev. Biochem. 47:251–276.
11. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotics resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69:2110–2114.
12. **Cronan, J. E., Jr., Y. Y. Chang, and C. Grabau.** 1985. Membrane lipoproteins in procaryotic and eucaryotic cells: a genetic approach to the study of protein-lipid interactions, p. 299–302. *In* L. Leive (ed.), Microbiology—1985. American Society for Microbiology, Washington, D. C.
13. **Cunningham, C. C., and L. P. Hager.** 1971. Crystalline pyruvate oxidase from *Escherichia coli*. III. Phospholipid as an allosteric effector for the enzyme. J. Biol. Chem. 246:1583–1589.
14. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. 112:295–298.
15. **Eisenberg, D.** 1984. Three-dimensional structure of membrane and surface proteins. Annu. Rev. Biochem. 53:595–623.
16. **Gennis, R. B., and L. P. Hager.** 1976. Pyruvate oxidase, p. 493–504. *In* A. Martonosi (ed.), The enzymes of biological membranes, vol. 2. Plenum Publishing Corp., New York.
17. **Grabau, C., and J. E. Cronan, Jr.** 1984. Molecular cloning of the gene (*poxB*) encoding the pyruvate oxidase of *Escherichia coli*, a lipid-activated enzyme. J. Bacteriol. 160:1088–1092.
18. **Koland, J. G., M. J. Miller, and R. B. Gennis.** 1984. Reconstitution of the membrane-bound, ubiquinone-dependent pyruvate oxidase respiratory chain of *Escherichia coli* with the cytochrome *d* terminal oxidase Biochemistry 23:445–453.
19. **Levitt, M.** 1981. Effect of proline residues on protein folding. J. Mol. Biol. 145:251–263.
20. **Lo, K.-M., S. S. Jones, N. R. Hackett, and H. G. Khorana.** 1984. Specific amino acid substitutions in bacterioopsin: replacement of a restriction fragment in the structural gene by synthetic DNA fragments containing altered codons. Proc. Natl. Acad. Sci. USA 81:2285–2289.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Maquat, L. E., and W. S. Reznikoff.** 1978. *In vitro* analysis of the *Escherichia coli* RNA polymerase interaction with wild-type and mutant lactose promoters. J. Mol. Biol. 125:467–490.
23. **Mather, M., R. Blake, J. Koland, H. Schrock, P. Russell, T. O'Brien, L. P. Hager, and R. B. Gennis.** 1982. *Escherichia coli* pyruvate oxidase: interaction of a peripheral membrane protein with lipids. Biophys. J. 37:87–88.
24. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. 101:20–78.
25. **O'Brien, T. A., R. Blake, and R. B. Gennis.** 1977. Regulation by lipids of cofactor binding to a peripheral membrane enzyme: binding of thiamin pyrophosphate to pyruvate oxidase. Biochemistry 16:3105–3109.
26. **Recny, M. A., and L. P. Hager.** 1983. Isolation and characterization of the protease-activated form of pyruvate oxidase. J. Biol. Chem. 258:5189–5195.
27. **Russell, P., L. P. Hager, and R. B. Gennis.** 1977. Lipid activation and protease activation of pyruvate oxidase. J. Biol. Chem. 252:7877–7882.
28. **Russell, P., H. L. Schrock, and R. B. Gennis.** 1977. Characterization of the proteolytic activation of pyruvate oxidase. J. Biol. Chem. 252:7883–7887.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
30. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. DNA 3:479–488.

JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1279–1289
0021-9193/82/091279-11$02.00/0

Vol. 151, No. 3

Mapping Nonselectable Genes of *Escherichia coli* by Using Transposon Tn*10*: Location of a Gene Affecting Pyruvate Oxidase

YING-YING CHANG AND JOHN E. CRONAN, JR.*
Department of Microbiology, University of Illinois, Urbana, Illinois 61801

Received 6 April 1982/Accepted 21 May 1982

Mutants of *Escherichia coli* K-12 deficient in pyruvate oxidase were isolated by screening for the production of $^{14}CO_2$ from [1-^{14}C]pyruvate by the method of Tabor et al. (J. Bacteriol. 128:485–486, 1976). One of these lesions (designated *poxA*) decreased the pyruvate oxidase activity to 10 to 15% of the normal level but grew well. To map this nonselectable mutation, we isolated strains having transposon Tn*10* inserted into the chromosome close to the *poxA* locus and mapped the transposon. These insertions were isolated by the following procedure: (i) pools of Tn*10* insertions into the chromosomes of two different Hfr strains were prepared by transposition from a λ::Tn*10* vector; (ii) these Tn*10*-carrying strains were then mated with a *poxA* recipient strain, and tetracycline-resistant (Tet^r) recombinants were selected; (iii) the Tet^r recombinants were then screened for $^{14}CO_2$ production from [1-^{14}C]pyruvate. This method was shown to give a >40-fold enrichment of insertions of Tn*10* near the *poxA* gene as compared with transduction. Calculations indicate that a similar enrichment should be expected for other genes. The enrichment is due to the much greater map interval over which strong linkage between selected and unselected markers is found in conjugational crosses as compared with transductional crosses. The use of Hfr conjugative transfer allows isolation of transposon insertions closely linked to a nonselectable gene by scoring hundreds rather than thousands of colonies. Using a Tn*10* insertion >98% cotransduced with the *poxA* locus, we mapped the *poxA* gene on the *E. coli* genetic map. The *poxA* locus is located at 94 min, close to the *psd* locus. The clockwise gene order is *ampA*, *poxA*, *psd*, *purA*. The *poxA* mutation is recessive and appears to be a regulatory gene.

The pyruvate oxidase of *Escherichia coli* is a peripheral membrane enzyme that catalyzes the oxidative decarboxylation of pyruvate to acetic acid plus CO_2 (16). The enzyme has been purified to homogeneity (40) and is a tetramer consisting of four identical polypeptide chains. Thiamine pyrophosphate and flavin adenine dinucleotide are required cofactors of the reaction (16). The activity of pyruvate oxidase increases 20-fold or more in the presence of various lipids or detergents, and these agents are active at very low concentrations (4, 12–14, 36). Pyruvate oxidase has a high-affinity binding site for lipid (36), and this enzyme-lipid interaction seems to increase activity by triggering a conformational change of the protein (36). A similar activation of the enzyme is observed upon controlled proteolysis of pyruvate oxidase (34). This protease-activated form of the oxidase is not further activated by lipid, and proteolysis results in loss of the high-affinity lipid binding site (35). These in vitro experiments indicate that pyruvate oxidase is an excellent enzyme for studying protein-lipid interaction.

Although the enzymological and physical characterization of *E. coli* pyruvate oxidase has been thorough, the physiological function of this enzyme is unclear. The major enzyme catalyzing the conversion of C3 compounds to C2 compounds (pyruvate to acetyl coenzyme A) in *E. coli* is pyruvate dehydrogenase (22, 25, 26). Mutants deficient in pyruvate dehydrogenase (*aceEF*) require an exogenous source of acetate for growth (22, 25, 26). Since the pyruvate oxidase activity of *E. coli* is substantial (similar to that of pyruvate dehydrogenase in crude extracts) and produces acetate, it would seem that a deficiency in pyruvate dehydrogenase should not elicit an acetate requirement. It has been suggested that the pyruvate oxidase activity is too low to produce sufficient acetate to support the growth of *ace* strains (22). However, it also seems possible that the rate of conversion of the free acetate produced by the oxidase to

TABLE 1. Bacterial strains

Strain	Relevant markers	Source (reference)[a]
KL333	HfrC, *leu-40*	CGSC4337
JRG596	F⁺ Δ(*aroP-aceEF*), *pps*	J. R. Guest (25)[b]
CY265	HfrC, Δ(*aroP-aceEF*)	P1 (JRG596) × KL333
CY314	λcI857 lysogen of CY265	This work
χ478	*thi-1*, *proC32*, *purE42*, *leuB6*, *metE70*, *trpE38*, *lysA23*, *rpsL109*, *lacZ36*, *ara-14*, *xyl-5*, *mtl-1*, *tsx-67*, *azi-6*, *supE45 tonA23*	B. Low
AB2880	*aroD6*	CGSC4869
PA505-1-5	*pps-4*	CGSC4341
KL290	*serC13*, *serS14*	M. Pascal (38)
CB898	*pfl-1*	CGSC5409
PC0950	*purA54*, *serB28*	CGSC5999
JB1	*uxuA1*	CGSC5671
CM8	*uxuB108*	CGSC5030
PC1	*dnaC1*	CGSC4141
AB4141	*valS7*	CGSC4812
H882	*purA*	E. Hawrot (20)
T850	*mop-1*	K. B. Low (27, 28)
EH450	*psd-2*, *ampA73*	CGSC4255
Hfr strains H, 6, KL16, P801, P4X, J4, Ra2	Hfr (Fig. 1)	CGSC4259
KLF17/KL132	F117 *purA*⁺ *pyrB*⁺/*pyrB*, *hisG*, *thyA*, *recA*	This work
KLF18/KL132	F118 *purA*⁺ *pyrB*⁺/*pyrB*, *hisG*, *thyA*, *recA*	This work
YYC7	*poxA1* of CY314	P1 (YYC16) × PA505-1-5
YYC16	*zdh*::Tn*10*, *aroD*⁺ of AB2880	This work
YYC21	*zdh*::Tn*10* of PA505-1-5	P1 (YYC33) × CB898
YYC33	*zca*::Tn*10*, *ser*⁺ of KL290	This work
YYC34	*zca*::Tn*10* of CB898	P1 (YYC34) × JRG596
YYC63	*pps-4*, *pfl-1* of CY265	This work
YYC65	*pfl-1* of JRG596	P1 (YYC85) × JRG596
YYC85	*zac*::Tn*10*, *ace*⁺ of YYC65	This work
YYC88	*zac*::Tn*10* of JRG596	P1 (YYC115) × EH450
YYC91	*rpsL* of YYC7	P1 (YYC115) × EH450
YYC107	*pps-4*, *pfl-1* of YYC7	This work
YYC109	*zje-2*::Tn*10*, *poxA*⁺ of YYC107	Heat-pulse curing
YYC115	*zje-1*::Tn*10*, *poxA*⁺ of YYC91	
YYC170	*zje-1*::Tn*10*, *psd*⁺ of EH450	
YYC176	*zje-1*::Tn*10*, EH450	
YYC177	Δ(*aroP-aceEF*), *poxA*, *zje-1*::Tn*10* of X478	
YYC183	YYC107 cured of λ prophage	

[a] Strains designated CGSC were obtained from the Coli Genetic Stock Center, Yale University, New Haven, Conn., through the kindness of the curator, B. Bachmann.

[b] Strain JRG596 has also been referred to as strain KΔ15 (25, 26). This strain is also deficient in pyruvate oxidase, but the site of this lesion is unknown. We failed to transduce this strain to *pox*⁺ with P1 phage grown on a *pox*⁺ strain. It should be noted that this strain was heavily mutagenized during its derivation. We also found that this strain is F⁺ rather than F⁻.

acetyl-coenzyme A could limit the utilization of this acetate source.

To approach problems of lipid-protein interaction by genetic means, we isolated mutants of *E. coli* K-12 deficient in pyruvate oxidase. The mutants were isolated by screening for $^{14}CO_2$ production from [1-^{14}C]pyruvate, using the method of Tabor et al. (37). Since these mutants have no selectable phenotype, we developed a method in which the transposon Tn*10* is used to map one class of these mutants, designated *poxA* (for pyruvate oxidase).

MATERIALS AND METHODS

Bacterial strains. All bacteria were derivatives of *E. coli* K-12 and are listed in Table 1. Strains for studying pyruvate oxidase have to be *ace* (lacking pyruvate dehydrogenase [16, 25]). An *ace* mutation was introduced by either of two methods. In the first method, *leu* strains (e.g., KL333) were transduced to *leu*⁺ with P1 phage grown on strain JRG596 to give the Δ*aceEF* derivatives (e.g., CY265). In the second method, strain YYC85, a strain with a Tn*10* insertion very close (>99% cotransduced) to the *ace* locus, was isolated by transducing strain YYC65 with P1 phage grown on a

pool of Tn*10* insertions into the *E. coli* chromosome (see below and Table 1). Strain YYC88 (Tet^r *ace*) was obtained by transducing strain JRG596 with P1 phage grown on strain YYC85. A P1 stock prepared from strain YYC88 was then used to construct Tet^r *ace* strains (e.g., YYC177).

Construction of *pps* strains was also done by using a closely linked Tn*10* insertion. The *pps* locus maps very close to the *aroD* locus at 37 min of the *E. coli* chromosome (1, 7). Strain AB2880 (*aroD*) was transduced by P1 phage grown on a pool of random Tn*10* insertions into the *E. coli* chromosome to give the Tet^r *aroD*$^+$ strain YYC16. Strain PA505-1-5 (*pps*) was transduced with P1 phage grown on strain YYC16, and Tet^r colonies were scored for *pps* (lack of growth with lactate as sole carbon source). A phage P1 stock grown on strain YYC21 (Tet^r *pps*) was then used to transduce strain CY265 or other strains to give *pps* derivatives (the cotransduction frequency of Tet^r and *pps* was ~67%).

The method for construction of *pfl* strains was analogous to that used for constructing *pps* strains. The *pfl* locus maps very close to the *serC* and *serS* loci, at 20 min of the *E. coli* chromosome (1, 38). Strain KL290 (*serC serS*) was transduced with P1 phage grown on a pool of random Tn*10* insertions into the chromosome to give the Tet^r *serC*$^+$ *serS*$^+$ strain YYC33. Strain CB898 (*pfl*) was transduced with P1 phage grown on strain YYC33, and Tet^r colonies were isolated and screened for the *pfl* phenotype. *pfl* colonies were identified by a soft agar overlay method (30). Soft agar (5 ml) containing 2.5 ml of 1.5% agar, 1.37 ml of 10% sodium pyruvate, 1 ml of 0.5% benzyl viologen, and 0.125 ml of 1 M potassium phosphate buffer (pH 7.0) was poured over the colonies to be screened. *pfl* strains gave white colonies in the soft agar overlay, whereas *pfl*$^+$ colonies were purple. The *zca*::Tn*10* insertion cotransduces ~60% with the *pfl* locus. Strains that had been made *pps* or *pfl* as described above had a Tn*10* insertion near the locus. The Tet^r was deleted by the method of Bochner et al. (5) as modified by Maloy and Nunn (29). These Tn*10*-deleted strains were then used again in crosses, with Tn*10* as the selected marker. In fact, several strains had been through several cycles of insertion and deletion of Tn*10* (e.g., strains YYC63 and YYC107; see Table 1). Strain YYC183 is strain YYC107 cured of its λ prophage by heat-pulse curing (17).

Media. Rich broth contained (in grams per liter): tryptone, 10; NaCl, 5; and yeast extract, 1 and was supplemented with 10 mM sodium acetate for all Δ*aceEF* strains. Rich agar media were rich broth plus 1.5% agar. Minimal media were medium E (31) or medium M9 (31) supplemented with thiamine (1 mg/liter). The carbon sources used (at 0.4%) were glucose, succinate, lactate, or D-glucuronic acid. L-Amino acids (50 mg/liter), casein hydrolysate (0.1%), nucleic acid bases (25 mg/liter), streptomycin (100 mg/liter), and tetracycline-hydrochloride (10 mg/liter) were added to media as indicated.

The triphenyltetrazolium chloride (TTC)-containing medium was that of Bochner and Savageau (6) containing 0.5% sodium pyruvate and 0.01% sodium acetate.

Chemicals. Sodium [1-^{14}C]pyruvate was a product of New England Nuclear Corp. *N*-Methyl-*N'*-nitrosonitrosoguanidine and 2,3,5-triphenyltetrazolium chloride monohydrate were products of Aldrich Chemical Co. Sodium ferricyanide was obtained from ICN Pharmaceuticals, Inc.; benzyl viologen and Triton X-100 were obtained from Sigma Chemical Co.; and hyamine was obtained from Packard Instrument Co.

Mutagenesis and screening of mutants. Strain CY314 (a strain lysogenic for λ*c*1857) was grown in medium E plus glucose and acetate until log phase and then mutagenized with *N*-methyl-*N'*-nitroso-nitrosoguanidine at a final concentration of 0.1 mg/ml for 30 min at 33°C (8, 9). The bacteria were washed once, suspended in the same medium, and grown overnight. Over 90% of the organisms were killed by the mutagenesis. The mutagenized cells were diluted and plated on minimal medium E containing glucose and acetate. Single colonies were isolated and screened on microtiter plates as described below.

Screening for pyruvate oxidase mutants was done on 96-well microtiter plates as described by Tabor et al. (37) with some modifications. A colony was inoculated into the well of a microtiter plate containing 0.1 ml of M9 medium (with the pH decreased to 6.0) plus 0.4% glucose and 2 mM acetate. After growing overnight at 33°C, the cultures were inoculated into a second microtiter plate with a 48-prong stamping device. The second microtiter plate contained 0.1 ml of the same medium, except the glucose concentration was growth limiting (0.02%). After these cultures were grown overnight at 33°C (whereupon growth was limited by glucose deficiency), a mixture of 40% glucose and 10% casein hydrolysate was added to each well with the stamping device (transferring ca. 3 μl per well). The plates were incubated at 33°C for 2 h and then shifted to 42°C for 3 h to induce the temperature-sensitive λ prophage and lyse the cells. To each well of these lysates was added 0.1 ml of a mixture of 0.2 M sodium phosphate buffer, pH 6.0, 20 mM $MgCl_2$, 0.2 mM sodium thiamine pyrophosphate, 10 mM [1-^{14}C]-pyruvate sodium salt (6.25×10^{-2} Ci/mol), and 16 mM $Na_3Fe(CN)_6$ (final concentrations are given). The plates were immediately covered with filter paper that had been impregnated with $Ba(OH)_2$ as described by Tabor et al. (37). The plate was incubated at 42°C for 4 to 5 h, and the filter paper cover was replaced by a second $Ba(OH)_2$-treated filter paper. The plate was then incubated at 42°C for 12 to 16 h. Both filter papers were autoradiographed (to give an exposure in the linear range of the film for most wells). A lack of exposure of the film above the position of a microwell on the autoradiogram indicated a possible pyruvate oxidase mutant.

Preparation of pools of random insertions of Tn*10* into the *E. coli* chromosome. Phage λNK370 was plaque purified twice, and a stock with a titer of 2×10^{10} to 3×10^{10}/ml was prepared (8, 9, 24). Pools of random insertions of Tn*10* from λNK370 into the chromosomes of three strains of *E. coli*, CY314, KL16, and YYC63, were prepared essentially as previously described (8, 9). The *E. coli* strains were grown overnight in tryptone broth supplemented with acetate and maltose. Approximately 10^{10} bacteria were infected with λNK370 at a multiplicity of 0.2. After incubation for 45 min, they were plated on rich media supplemented with acetate, 2.5 mM sodium pyrophosphate, and 20 mg of tetracycline-hydrochloride per liter. After 2 to 3 days, individual Tet^r colonies appeared. A total of 2,000 to 4,000 independent Tet^r

colonies of each strain were pooled by washing the colonies from the plates with medium E. The pooled colonies of the random insertions of Tn*10* into strains CY314 and KL16 were used for conjugation experiments as described below. A P1 stock was prepared on the pooled colonies of strain YYC63, which was used for experiments involving the indicator medium.

A pool of random insertion of Tn*10* into the chromosome of the wild-type strain W1485 was prepared as described previously by A. Klages Ulrich of this laboratory (8, 9). P1 phage grown on this pool was used in experiments to isolate strains YYC85 (*zac*::Tn*10*), YYC16 (*zdh*::Tn*10*), and YYC33 (*zca*::Tn*10*) as described above.

Screening *pox* mutants on TTC-containing medium. Screening on TTC-containing medium is reliable only when <100 colonies are present per plate. This behavior upon crowding (6) therefore precluded scoring by replica plating onto TTC-containing medium. Another problem was that the presence of tetracycline interfered with scoring on TTC medium. For these reasons, the screening of *poxA* mutants on TTC medium was usually done by either of the following two methods. In the first method, all colonies (e.g., Tet^r colonies) to be screened were eluted from plates by washing the entire plate with medium E. This mixture of colonies was then diluted and plated on TTC medium to give approximately 100 colonies. The *pox* colonies were recognized as white colonies (in an *ace pps pfl* genetic background) after 2 days of growth (the presence of any pyruvate-utilizing enzyme gives a red colony on this indicator medium). In the second method, the colonies to be screened were gridded onto master plates. Each colony was then inoculated into a well of a 96-well microtiter plate containing 0.1 ml of medium E supplemented with 0.4% glucose and acetate. After growth overnight, the stamping device was inserted into the first microtiter plate and used to inoculate a second microtiter plate containing the same medium. The cultures in the second plate were allowed to grow overnight. The contents of each well were then serially diluted with the stamping device into a series of microtiter plates containing medium E. After proper dilution, the stamping device delivered only a few bacteria per prong onto the TTC medium. This method avoided artifacts of crowding and allowed scoring of ordered arrays of colonies.

Preparation and assay of pyruvate oxidase. Strains of *E. coli* grown to early stationary phase in rich broth supplemented with acetate were harvested and washed once with 0.02 M sodium phosphate buffer (pH 6.0). This same buffer was added to the pellet (2 ml/g [wet weight]), and the suspension was treated at full power of an ultrasonicator fitted with a small probe (50% pulse) for 3 min for each 5 ml of suspension. The suspension was centrifuged at 40,000 × *g* for 1 h, and the supernatant was assayed. All operations were done at 0 to 4°C. The activity of 40,000 × *g* supernatant was stable for at least 1 month if stored at −7°C in the presence of 20% glycerol (4, 34).

Two methods were used to measure pyruvate oxidase activity. Similar results were obtained with either assay to determine the activities of crude extracts of either wild-type or mutant cultures. In the spectrophotometric method, the assay was carried out in 1-ml cuvettes in a Gilford spectrophotometer. Extract (≤0.1 ml) was added to 0.8 ml of an assay mixture consisting of 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.0), 0.05 ml of 0.2 M $MgCl_2$, 0.05 ml of 2 mM sodium thiamine pyrophosphate, and 0.2 ml of 1 M sodium pyruvate. The mixture was incubated at room temperature for 20 min. Then, 0.1 ml of 0.08 M $Na_3Fe(CN)_6$ was added, and the rate of decrease of the absorbance at 450 nm was recorded (4). The enzymatic activity was calculated by using the formula $E^{450}_{mM} = 0.218\ cm^{-1}$ and is expressed as the amount of pyruvate consumed per unit of time, assuming 2 equivalents of ferricyanide are reduced per equivalent of pyruvate decarboxylated (4).

In the radioactive method, the reaction mixture (0.1 ml) consisted of 0.05 ml of 0.2 M sodium phosphate buffer (pH 6.0), 5 μl of 0.2 M $MgCl_2$, 5 μl of 2 mM sodium thiamine pyrophosphate, 5 μl of 1 M sodium [1-^{14}C]pyruvate (6.25×10^{-2} Ci/mol), 10 μl of 0.08 M $Na_3Fe(CN)_6$, and the extract to be assayed. The assay mixture in an Eppendorf vial was placed in a liquid scintillation counting vial as described previously (3). Filter paper (3.5 by 2.5 cm) soaked with 0.15 ml of 1 M Hyamine hydroxide lined the wall of the counting vials to trap the $^{14}CO_2$. After incubating at 33°C for 20 min with shaking, 0.05 ml of 3 N HCl was added to the assay mixture, and incubation was continued for 30 min. After collection, the Eppendorf vials were discarded, 15 ml of toluene scintillation fluid was added to the scintillation vial, and the vials were counted.

Other methods. Protein concentrations were determined by a microbiuret procedure with bovine serum albumin as standard (32). Transductions with P1 *vir* were performed as previously described (11).

Calculations of genetic linkage. Wu (41) has derived the following equation to relate cotransduction frequency with phage P1 to the distance between two markers on the *E. coli* chromosome: $F = (1 - d/L)^3$, where F is the cotransduction frequency, d is the distance between the two markers, and L is the length of the transduced DNA fragment. For 90% cotransduction ($F = 0.9$) and $L = 2.3$ min (the length of DNA packaged by phage P1), $d = 0.07$ min.

An analogous equation relating linkage of unselected and selected markers in prolonged Hfr × F^- conjugational crosses has been derived by de Haan and Verhoef (15, 39): $(1 - \beta) = (1 - \alpha)(1 - e^{-ku})$, where β is the frequency of an unselected marker, α is the incorporation frequency of a given marker, k is the number of breakages per map minute, and u is the distance (in minutes) between the selected and unselected markers. For a linkage of 90% between the selected and unselected markers where α is 0.5 (15, 39) and k is 0.2 (average of the values given in reference 39), $u = 1.1$ min.

The probability of insertion of a transposon into a unique fragment of DNA (that adjacent to the nonselectable marker) was calculated from the following equation of Clarke and Carbon (10): $N = \ln(1 - P)/\ln(1 - f)$, where N is the number of Tet^r recombinants required to detect a transposon ≥90% linked to the nonselectable marker, P is the probability of Tn*10* insertion into a unique DNA sequence, and f is the fraction of the genome into which insertion is required. Since the genome of *E. coli* is 100 min (1, 2), f for ≥90% cotransduction is 0.07/100 = 0.0007 and P = 0.99; N = 6,576. In contrast, in the conjugational

cross, $f = 1.12/100 = 0.0112$. When P is again 0.99, $N = 409$.

This analysis assumes that Tn*10* acts genetically as a point mutation. This assumption appears to be justified (24). However, the assumed random nature of Tn*10* insertion is not accurate (see below).

RESULTS

Characterization of pyruvate oxidase of *E. coli* K-12. The previous work on pyruvate oxidase was on the enzyme from *E. coli* W191-6 (16), a derivative of strain W. Since strain W is genetically cryptic, we used *E. coli* K-12. The properties of pyruvate oxidase from *E. coli* K-12 were found to be very similar to those of pyruvate oxidase from strain W191-6. The activity in the crude extract from the *E. coli* K-12 strain CY314 is dependent on the presence of pyruvate, $MgCl_2$, and thiamine pyrophosphate and is inhibited by the antibody prepared against *E. coli* W191-6 pyruvate oxidase (a gift of R. Kranz and R. Gennis). Detergents such as Triton X-100 increase the activity in the crude extracts somewhat, but the activation is not as dramatic as that of the purified enzyme from strain W191-6 (16). This was attributed to activation by the phospholipids present in the extracts.

The pyruvate oxidase activity of *E. coli* K-12 increased during the growth of the bacterial cultures and reached maximum activity during early stationary phase. The activity was unchanged by growth through late stationary phase. No detectable pyruvate oxidase activity was found when strain CY314 was grown under anaerobic conditions with or without sodium nitrate as an electron acceptor.

Isolation and characterization of *pox* mutants. Tabor et al. (37) have reported a method that permits the rapid screening of a large number of bacterial colonies for mutations in pathways producing $^{14}CO_2$. An assay is done on single colonies, each in the well of a microtiter dish, by trapping the $^{14}CO_2$ from each well on a filter paper saturated with barium hydroxide. We utilized this method, with some modifications, as the basis for isolation of pyruvate oxidase mutants (see Materials and Methods). The major modification was that we assayed pyruvate oxidase activity in lysates produced by inducing a phage λ lysogen.

Strain CY314 was mutagenized with *N*-methyl-*N'*-nitroso-nitrosoguanidine, and approximately 1,000 mutagenized colonies were screened by the microtiter plate method. Several mutant candidates were obtained, and they were further characterized by assay of their pyruvate oxidase activities in crude extracts. Most of the candidates had about half of the pyruvate oxidase activity of the parent strain. Only one mutant, YYC7, had a lower activity (about 15% of the parent strain [Table 2]). Thus, we concentrated our effort in characterizing and mapping

TABLE 2. Pyruvate oxidase activities of various strains

Strain	Genotype	Activity (U/mg of protein)[a]	K_m for pyruvate (mM)[b]
Expt 1			
CY314	Wild type	135	14 (33)
YYC7	*poxA1*	16	17 (25)
Expt 2			
CY314	Wild type	113	
YYC7	*poxA1*	16	
CY314 + YYC7[c]		137	
Expt 3			
CY314	Wild type	152	
YYC7	*poxA1*	18	
YYC91	*poxA1*	23	
YYC115	*poxA*$^+$ of YYC91	183	
YYC265	Wild type	141	
YYC63	*pfl pps* of CY265	70	
YYC107	*pfl pps poxA1*	<2	
YYC109	*poxA*$^+$ of YYC107	78	

[a] The spectrophotometric assay was used. One unit of activity equals 1 nmol of pyruvate decarboxylated per min.

[b] The K_m value for pyruvate as determined by spectrophotometric or (values in parentheses) radioactive assay. The differences between the two assays probably reflects a lower extent of activation by endogenous phospholipid in the radioactive assay.

[c] Equal amounts of protein from the two extracts were mixed and assayed.

this mutant, which we designated *poxA* (for pyruvate oxidase).

Although strain YYC7 was deficient in pyruvate oxidase activity, mutant extracts retain significant amounts of oxidase activity (10 to 15% of the normal level). Preliminary experiments indicate that the level of pyruvate oxidase antigen in mutant extracts is similarly decreased. Since the residual activity in the *poxA* strains has a normal Michaelis constant for pyruvate (Table 2), strain YYC7 seems to contain an abnormally low level of a structurally normal pyruvate oxidase. When an extract of strain YYC7 was mixed with an extract of the parent strain, CY314, there was no inhibition of the pyruvate oxidase activity of the latter extract, indicating that no inhibitor was present in the extract of strain YYC7 (Table 2).

Mutant YYC7 grows as well as the parent strain, but at a slightly slower rate. The generation times of strains CY314 and YYC7 were 55 and 95 min, respectively, when grown on rich broth supplemented with acetate at 33°C. On glucose minimal medium supplemented with acetate, strain YYC7 doubled in 115 min, somewhat slower than strain CY314 (100 min). This low growth rate seemed characteristic of the *poxA* lesion, since *poxA* transductants of other strains also grew more slowly than the parental strain.

Insertions of Tn*10* near the *poxA* gene, nonselectable gene. The mapping of the *poxA* gene was complicated by its nonselectable phenotype and by the fact that reliable screening for the enzyme activity required pyruvate dehydrogenase-deficient strains (the assay for the pyruvate decarboxylase component of the dehydrogenase is very similar to that of pyruvate oxidase). We therefore chose to isolate an insertion of the transposon Tn*10* close to the *poxA* gene and map the gene by locating the site of insertion. This is a method put forth by Kleckner and co-workers (24), who also suggested that appropriate insertions could be readily isolated for nonselectable as well as selectable genes. The latter case is clearly true and has been used in a number of laboratories, including our own. However, few nonselectable genes have been mapped by this procedure, owing to the difficulty in isolating closely linked insertions. Calculations (see Materials and Methods) indicate that isolation of such an insertion would require screening of several thousand Tet^r recombinants. Hence, a screening method such as an indicator medium would suffice, whereas a method such as assaying CO_2 evolution of microtiter plate cultures would be most laborious.

We therefore chose to enrich for Tn*10* insertions close to the *poxA* gene by screening Tet^r recombinants of crosses between Tn*10*-carrying *poxA*$^+$ Hfr strains and a *poxA* recipient strain (an F$^-$ phenocopy culture of strain YYC91). The Hfr cultures contain a mixture of several thousand strains, each with a Tn*10* insertion of independent origin. Two Hfr strains having different transfer origins (HfrC and KL16) were used to cover the entire chromosome, and prolonged matings were done. Tet^r recombinants were selected and screened for $^{14}CO_2$ evolution by the microtiter dish assay. Owing to the greater length of DNA transferred in such crosses, Tet^r *pox*$^+$ recombinants were isolated at a much greater frequency than that expected from phage P1 transduction from a pool of Tn*10* insertions.

Using this method, we screened about 180 recombinants each from crosses of Hfr strains C and KL16 with a *poxA* recipient (strain YYC91). In the HfrC cross, about 25% of the Tet^r recombinants were *pox*$^+$, whereas no *pox*$^+$ recombinants were found in the cross with strain KL16. From our later experiments in which an indicator plate method was used to screen for *pox*$^+$ (see below), a linkage frequency of 0.6% would have been expected if the experiment had been done by transduction rather than conjugation.

The *pox*$^+$ Tn*10* strains from the conjugational crosses were then screened for those with Tn*10* to *poxA* linkages sufficiently high to be cotransduced. A P1 stock was grown on a pool of these strains. A *poxA* strain was transduced to Tet^r, and the Tet^r transductants were again screened by the microtiter plate method. About 10% of the Tet^r transductants (180 screened) were *pox*$^+$, and several were shown to have a normal level of pyruvate oxidase by the spectrophotometric assay (Table 2). One of these insertions, *zje-1*:Tn*10*, which subsequent transductions showed to be tightly linked (98%) to *poxA*, was used to locate the gene on the genetic map.

Insertions of Tn*10* near the *poxA* gene were later isolated directly by cotransduction. We found that we could score the presence of pyruvate oxidase in individual colonies by using a pyruvate-TTC indicator medium, provided the strains were deficient in all other pyruvate-utilizing enzymes (*ace*, *pps*, *pfl*). Although introduction of the *pfl* mutation into *pox*$^+$ strains decreased pyruvate oxidase levels by about 50%, this did not affect screening for *pox*$^+$ recombinants. We used the indicator medium to screen about 3,600 Tet^r colonies from a transduction of strain YYC107 with a phage P1 stock grown on a pool of Tn*10* insertions into strain YYC63. We isolated 22 presumably independent Tn*10* insertions cotransduced with the *poxA* locus. One of these insertions, *zje-2*::Tn*10*, 98% linked to *poxA*, was used in some mapping experiments. The site of this insertion seems to be identical to that of *zje-1*::Tn*10* (see below).

Mapping of the *poxA* gene. The conjugation

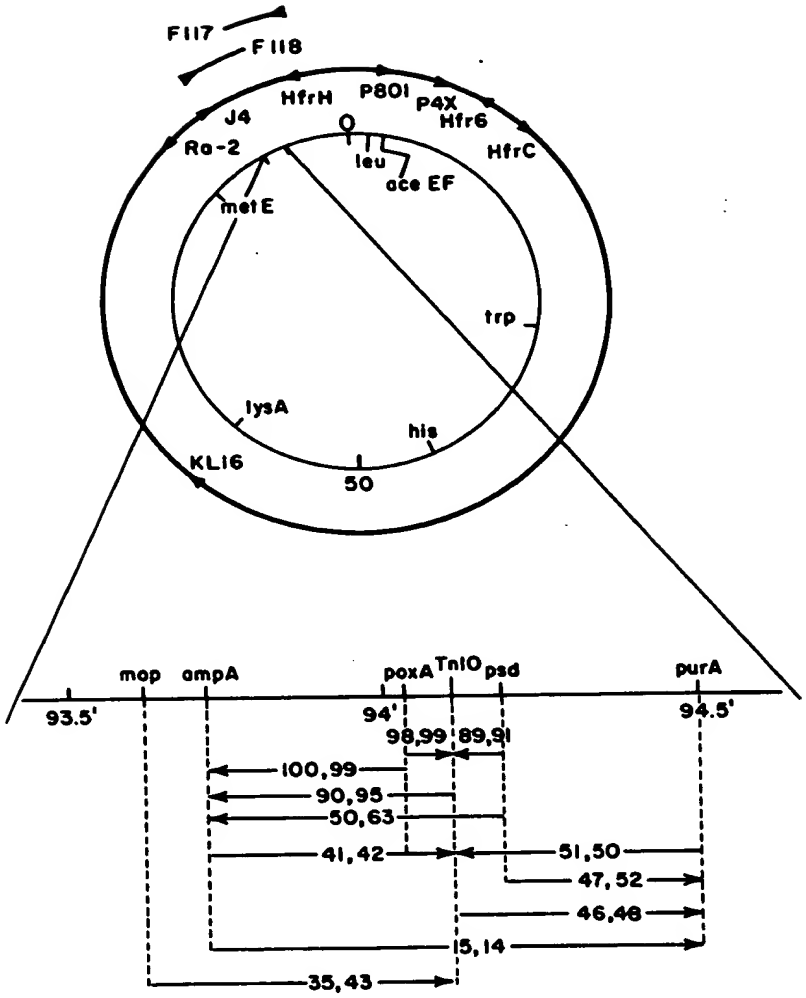


FIG. 1. Relevant portion of the *E. coli* genetic map. The linkages given are percent average cotransduction frequencies taken from experiments similar to those shown in Table 3. The first value is the cotransduction frequency with *zje2*::Tn*10* strain YYC109, and the second value is that with *zje1*::Tn*10* strain YYC115. The map is a modification of that of Bachmann and Low (1).

experiment described above already showed that the *poxA* locus was located between the origins of Hfr strains C and KL16 on the *E. coli* chromosome. Moreover, conjugation between the HfrC strain YYC109 and the F⁻ strain χ478 (which has markers spaced around the chromosome) showed that the Tn*10* insertion of the donor strain was most closely linked to *leu* (72%). To further delineate the location of *poxA*, a number of Hfr strains (27, 28) in this region were used (Fig. 1). Each Hfr strain was first transduced to Tet^r with phage P1 stock grown on strain YYC109 and then mated with the F⁻ strain χ478 for a short time (30 min), and Tet^r recombinants were selected. Only the derivatives of Hfr strains P4X, P801, and Ra-2 gave Tet^r recombinants. Identical results were obtained with the Tn*10* insertion of strain YYC115.

P1 transduction was used to more finely locate the Tn*10* insertions of strains YYC109 and YYC115. Strains carrying readily scorable markers in this region were transduced with phage P1 stocks grown on strains YYC109 or YYC115, and Tet^r recombinants were selected. Neither Tn*10* insertion cotransduced with *serB* (<0.7%), *dnaC* (<0.5%), *uxuAB* (<1%), *valS*

TABLE 3. Cotransductional mapping

Cross	P1 donor and relevant marker[a]	Recipient	Marker selected	No. of colonies scored	% Cotransduction frequency
1	YYC115 *zje-1*::Tn*10*	PC0950 *purA*	Tet^r	196	48
2	YYC115 *zje-1*::Tn*10*	H882 *purA*	Tet^r	200	49
3	YYC115 *zje-1*::Tn*10*	T850 *mop*	Tet^r	43	42
4	YYC115 *zje-1*::Tn*10*	EH450 *ampA*	Tet^r	96	45
5	YYC115 *zje-1*::Tn*10*	EH450 *psd*	Tet^r	96	95
6	YYC115 *zje-1*::Tn*10*	YYC7 *poxA*	Tet^r	89	95
7	YYC115 *zje-1*::Tn*10*	YYC107 *poxA*	Tet^r	48	100
8	YYC109 *zje-2*::Tn*10*	YYC107 *poxA*	Tet^r	48	98
9	YYC109 *zje-2*::Tn*10*	YYC107 *poxA*	Tet^r	200	98
10	YYC176 *zje-1*::Tn*10 ampA*	YYC183	*ampA*	143	95
11	YYC170 *ampA*	H882 *purA*	*pur*$^+$	144	11
12	EH450 *psd ampA*	YYC183	*ampA*	120	64

[a] The inheritance of the *pox*$^+$ allele of the donor was scored by the $^{14}CO_2$ microtiter dish assay (cross 6), by indicator plates with method 1 (cross 9) or method 2 (crosses 7 and 8). Only a few of the data obtained with strains carrying the *zje-2*::Tn*10* marker are shown. Crosses with these strains were done in parallel with those containing *zje-1*::Tn*10* insertion, and a similar number of transductants were scored. The cotransduction frequencies found for the two insertions to the other markers were essentially indistinguishable (see Fig. 1).

(<0.4%), or *metA* (<3%), but both insertions cotransduced with *purA* at a frequency of about 50% (Table 3).

Further transduction experiments showed cotransduction of both insertions with the *ampA* and *psd* loci (Table 3). The relative cotransduction frequencies suggested that both Tn*10* insertions were located between the *ampA* and *purA* loci, very close to the *psd* locus. A location between *ampA* and *purA* was demonstrated by three-factor transductional crosses (data not shown). Other three-factor crosses were consistent only with the order *ampA*, Tn*10*, *psd*, *purA* (Table 4). The Tn*10* insertions of strains YYC109 and YYC115 were indistinguishable in the crosses performed.

We then mapped the *poxA* locus in relation to the Tn*10* insertion and *psd* locus. Unfortunately, *psd* is a poor selected marker, owing to residual growth at 42°C, and thus we used Tn*10* and *ampA* as the selected markers in these crosses. When Tet^r was the selected marker in a transductional cross with strain YYC176 as the donor and strain YYC183 as the recipient, close linkage to both *poxA* (99%) and *psd* (86%) was found. There was no clear four-crossover class, and thus these data (not shown) suggested the map order *poxA*, Tn*10*, *psd*. Upon scoring the *poxA* markers of the recipient in cross 1 of Table 4, the linkage between *ampA* and *poxA* was 99%, whereas the cotransduction frequencies for both the Tn*10* insertion and the *psd* locus were lower (95 and 63%, respectively). These data are consistent only with the order *ampA*, *poxA*, Tn*10*, *psd*. The large differences in the cotransduction frequency observed between *ampA* and the Tn*10* insertion when the selected marker was shifted from one antibiotic to the other (42% when Tet^r was selected, 95% when *ampA* was selected) has been well documented

TABLE 4. Ordering of *ampA psd* and *zje1*::Tn*10*[a]

Cross	Donor	Recipient	Marker selected	No. of colonies scored	Class	% Frequency
1	YYC176 *zje-1*::Tn*10 psd ampA*	YYC183	*ampA*	143	Tet^r *psd*$^+$	32
					Tet^r *psd*	63
					Tet^s *psd*$^+$	5
					Tet^s *psd*	0
2	YYC115 *zje-1*::Tn*10*	EH450 *psd ampA*	Tet^r	96	*amp*$^+$ *psd*$^+$	42
					amp$^+$ *psd*	3
					ampA psd$^+$	53
					ampA psd	2

[a] In cross 1, the Tet^s *psd* recombinant class is taken as the four-crossover class, indicating the order *ampA*, Tn*10*, *psd*. The lack of a clear four-crossover class in cross 2 is consistent with this order.

with *ampA* (20) as well as other markers (2) and can be explained by the existence of sites within the *E. coli* chromosome that lead to preferential packaging into phage particles (2).

Diploidy at the *poxA* locus. Two F' strains, F117 and F118, which carry the chromosomal regions from 93 to 98 and 91 to 95 min of the *E. coli* chromosome, respectively (Fig. 1), were transferred into strain YYC177 (a Δ*aceEF*, *poxA* derivative of χ478). The resulting merodiploid strains and the recipient strain YYC177 were grown in liquid on a medium lacking histidine and thymine to select against the donor strains. Extracts of both merodiploid cultures had the same pyruvate oxidase activity as that of a wild-type strain, whereas the activity of monoploid strain YYC177 was very low. Since 60% of the cells in the merodiploid cultures were able to transfer *purA*$^+$ to strain H882, most of the cells were merodiploids rather than recombinants. These results indicated that the *poxA*$^+$ gene is carried by both F117 and F118 and that the *poxA* mutation is recessive to the wild-type allele.

DISCUSSION

Although quite deficient in pyruvate oxidase activity, the *poxA* mutant we isolated does not appear to be a mutant in the structural gene for this enzyme. We have recently isolated a second class of *pox* mutants (designated *poxB*) that preliminary results suggest are lesions in the structural gene for this protein. At this time, we therefore assign a regulatory function to the *poxA* gene. It is of interest that the level of pyruvate oxidase activity was decreased by introduction of the *pfl* mutation (Table 2). It may be that the *pfl* mutant (38) is a lesion in a regulatory gene rather than a structural gene for this complex enzyme. We also noted that *pfl*$^+$ *poxA aceEF* strains gave red colonies on the TTC indicator medium, indicating that pyruvate lyase functions in aerobically growing cells, a result in disagreement with a previous report (21).

Kleckner and co-workers (24) have pointed out that nonselectable genes can be readily mapped by isolation of a strain carrying a Tn*10* insertion near the gene of interest. The location of the transposon is mapped, and when the gene is mapped in relation to the transposon (e.g., by enzyme activity or protein profile), the gene can be accurately placed on the genetic map. However, the major difficulty lies in the isolation of a Tn*10* insertion close to the gene. Kleckner and co-workers (24) have implied that a transducing phage lysate of a pool of Tn*10* insertions into a wild-type strain could be used to transduce a strain defective in a nonselectable gene and the resulting Tet^r strains screened for the wild-type allele of the nonselectable gene. This is true, but calculations (see Materials and Methods) indicate that a powerful and facile screening method is needed. Assuming that Tn*10* inserts at random, the probability of a Tn*10* insertion cotransducible with a given gene is very low. For phage P1 transduction of *E. coli*, approximately 6,600 colonies having independent Tn*10* insertions must be screened to isolate a single insertion ≥90% cotransduced with a given gene (P = 0.99). Hence, although indicator plates would suffice, screening methods such as the microtiter dish $^{14}CO_2$ evolution assay of Tabor et al. (37) that can readily screen only a few hundred colonies are unfeasible. Since it seems likely that for many of the remaining unexplored genes of *E. coli* only rather laborious and expensive screens will be available, a method more powerful than direct screening of transductional Tn*10* recombinants seems desirable.

The key manipulation of our method is transfer of Tn*10* insertion pools via Hfr mating (rather than transduction) into an F$^-$ strain carrying a scorable allele of the nonselectable marker. During prolonged Hfr × F$^-$ matings, unselected markers are inherited by the recipient strain at a probability depending only on the map distance between the selected and nonselected markers (15, 39). The frequency of inheritance of nonselected markers is quite high. From the data and equations of de Haan and Verhoef (15, 39), markers separated by 1.1 min of the map inherit a ≥90% frequency (see Materials and Methods). Thus, isolation of a Tn*10* insertion close to a given nonselectable gene would require screening only 400 colonies (P = 0.99), an enrichment of about 20-fold as compared with a direct screening of transductants. The F$^-$ Tn*10* recombinants that inherit the Hfr allele of the gene of interest can then be pooled, and a transducing phage stock can be grown on this enriched pool and used in a subsequent screening for a highly cotransducible insertion.

A potential difficulty with our method concerns the gradient of chromosome transfer by Hfr strains. Although the gradient of transfer does not affect linkage between selected and nonselected markers separated by distances of <20 min (39), it does affect the efficiency of transfer of the selected marker. Thus, transposon insertions into chromosome segments transferred early will be preferentially found in the population to be screened. In our experiments, a high frequency of linkage was found despite a distance of 20 min between the HfrC origin and the location of the *poxA* gene, but more distal markers have not been tested. One solution to this problem would be the use of more than two Hfr strains; this, however, would increase the number of colonies to be screened. A better

solution would be to alleviate the transfer gradient by immobilization of mating complexes on a membrane filter (19). This procedure would decrease nonrandom transposon transfer to about the level observed in P1 transduction. Phage P1 preferentially transduces those markers close to the origin of chromosome replication (33).

We observed that the frequency of Tn*10* insertions near *poxA* was much higher than that calculated for both the conjugational and transductional cases. However, this was not unexpected, since the calculations assumed Tn*10* insertion to be at random and insertion of this transposon is known to be sequence specific (18, 23). About 90% of Tn*10* insertions are into "hot spots," DNA containing a specific 6-base-pair (GCTNAGC) sequence, whereas the remaining insertions are into DNA having degenerate versions of this sequence (18, 23). The high frequency with which we isolated Tn*10* insertions close to the *poxA* gene and the fact that two independently isolated insertions are genetically indistinguishable indicate that a hot spot for insertion of Tn*10* is located in this area of the chromosome. However, it should be noted that the presence of a hot spot should affect the conjugational and transductional crosses in a similar manner, and thus the relative enrichment of *poxA*-linked Tn*10* insertions predicted by the calculations should be valid. Indeed, a reasonable agreement is seen between the predicted and observed enrichments. Our calculations predict a 15- to 20-fold enrichment, whereas an enrichment of 40-fold was observed. The enrichment should suffice to make this procedure applicable to many nonselectable genes and to highly revertable selectable genes. Its use need not be restricted to Tn*10* and *E. coli* but should also be useful with other transposons and other conjugative bacteria.

ACKNOWLEDGMENTS

We thank David Clark for valuable discussion and S. J. Chang for support and encouragement.

This work was supported by National Science Foundation grant PCM 79-25689.

LITERATURE CITED

1. Bachmann, B. J., and K. B. Low. 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. 44:1–56.
2. Bachmann, B. J., K. B. Low, and A. L. Taylor. 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. 40:116–167.
3. Beaven, M., G. Wilcox, and G. K. Terpstra. 1978. A microprocedure for the measurement of $^{14}CO_2$ release from [^{14}C]carboxyl-labeled amino acids. Anal. Biochem. 84:638–641.
4. Blake, R., II, L. P. Hager, and R. B. Gennis. 1978. Activation of pyruvate oxidase by monomeric and micellar amphiphiles. J. Biol. Chem. 253:1963–1971.
5. Bochner, B. R., H. Huang, G. L. Schieven, and B. N. Ames. 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. 143:926–933.
6. Bochner, B. R., and M. A. Savageau. 1977. Generalized indicator plate for genetic, metabolic and taxonomic studies with microorganisms. Appl. Environ. Microbiol. 33:434–444.
7. Brice, C. B., and H. L. Kornberg. 1967. Location on the chromosome of *Escherichia coli* of a gene specifying phosphopyruvate synthase activity. Biochim. Biophys. Acta 136:412–414.
8. Clark, D., and J. E. Cronan, Jr. 1980. *Escherichia coli* mutants with altered control of alcohol dehydrogenase and nitrate reductase. J. Bacteriol. 141:177–183.
9. Clark, D., and J. E. Cronan, Jr. 1980. Acetaldehyde coenzyme A dehydrogenase of *Escherichia coli*. J. Bacteriol. 144:179–184.
10. Clarke, L., and J. Carbon. 1976. A colony bank containing synthetic col E_1 hybrid plasmids representative of the entire *E. coli* genome. Cell 9:91–99.
11. Cronan, J. E., Jr., D. F. Silbert, and D. L. Wulff. 1972: Mapping of the *fabA* locus for unsaturated fatty acid biosynthesis in *Escherichia coli*. J. Bacteriol. 112:206–211.
12. Cunningham, C. C., and L. P. Hager. 1971. Crystalline pyruvate oxidase from *Escherichia coli*. II. Activation by phospholipids. J. Biol. Chem. 246:1575–1582.
13. Cunningham, C. C., and L. P. Hager. 1971. Crystalline pyruvate oxidase from *Escherichia coli*. III. Phospholipid as an allosteric effector for the enzyme. J. Biol. Chem. 246:1583–1589.
14. Cunningham, C. C., and L. P. Hager. 1975. Reactivation of the lipid-depleted pyruvate oxidase system from *Escherichia coli* with cell envelope neutral lipids. J. Biol. Chem. 250:7139–7146.
15. de Haan, P. G., and C. Verhoef. 1966. Genetic recombination in *Escherichia coli*. II. Calculation of incorporation frequency and relative map distances by recombinant analysis. Mutat. Res. 3:111–117.
16. Gennis, R. B., and L. P. Hager. 1976. Pyruvate oxidase, p. 493–504. *In* A. Martonosi (ed.), The enzymes of biological membranes, vol. 2. Plenum Publishing Corp., New York.
17. Gottesman, M. E., and M. B. Yarmolinsky. 1968. The integration and excision of the bacteriophage lambda genome. Cold Spring Harbor Symp. Quant. Biol. 33:735–747.
18. Halling, S. M., and N. Kleckner. 1982. A symmetrical six-base-pair target site sequence determines Tn*10* insertion specificity. Cell 28:155–163.
19. Havekes, L. M., and W. P. M. Hoekstra. 1976. Characterization of an *Escherichia coli* K-12 F^- Con^- mutant. J. Bacteriol. 126:593–600.
20. Hawrot, E., and E. P. Kennedy. 1976. Conditional lethal phosphatidylserine decarboxylase mutants of *Escherichia coli*. Mol. Gen. Genet. 148:271–279.
21. Henning, U. 1963. Ein Regulationsmechanismus beim Abbau der brenztraubensaure durch *Escherichia coli*. Biochem. Z. 337:490–504.
22. Henning, U., and C. Herz. 1964. Ein Strukturgen-Komplex fur den Pyruvat-Dehydrogenase-Komplex von *Escherichia coli* K-12. Z. Vererbungsl. 95:260–275.
23. Kleckner, N. 1981. Transposable elements in prokaryotes. Annu. Rev. Genet. 15:341–404.
24. Kleckner, N., J. Roth, and D. Botstein. 1977. Genetic engineering *in vivo* using translocatable drug-resistance elements: new methods in bacterial genetics. J. Mol. Biol. 116:125–159.
25. Langley, D., and J. R. Guest. 1977. Biochemical genetics of the β-keto acid dehydrogenase complexes of *Escherichia coli* K12: isolation and biochemical properties of deletion mutants. J. Gen. Microbiol. 99:263–276.
26. Langley, D., and J. R. Guest. 1978. Biochemical genetics of the β-keto acid dehydrogenase complexes of *Escherichia coli* K12: genetics characterization and regulatory properties of deletion mutants. J. Gen. Microbiol. 106:103–117.

27. **Low, B.** 1973. Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. J. Bacteriol. 113:798–812.
28. **Low, K. B.** 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. 36:587–607.
29. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. 145:1110–1112.
30. **Mandrand-Berthelot, M., M. Y. K. Wee, and B. A. Haddock.** 1978. An improved method for the identification and characterization of mutants of *Escherichia coli* deficient in formate dehydrogenase activity. FEMS Microbiol. Lett. 4:37–40.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Munkres, K. D., and F. M. Richards.** 1965. The purification and properties of *Neurospora* malate dehydrogenase. Arch. Biochem. Biophys. 109:466–479.
33. **Newman, B. J., and M. Masters.** 1980. The variation in frequency with which markers are transduced by phage P_1 is primarily a result of discrimination during recombination. Mol. Gen. Genet. 180:585–589.
34. **Russell, P., L. P. Hager, and R. B. Gennis.** 1977. Characterization of the proteolytic activation of pyruvate oxidase. J. Biol. Chem. 252:7877–7882.
35. **Russell, P., H. L. Schrock, and R. B. Gennis.** 1977. Lipid activation and protease activation of pyruvate oxidase. J. Biol. Chem. 252:7883–7887.
36. **Schrock, H. L., and R. B. Gennis.** 1977. High affinity lipid binding sites on the peripheral membrane enzyme pyruvate oxidase. J. Biol. Chem. 252:5990–5995.
37. **Tabor, H., C. W. Tabor, and E. W. Hafner.** 1976. Convenient method for detecting $^{14}CO_2$ in multiple samples: application to rapid screening for mutants. J. Bacteriol. 128:485–486.
38. **Varenne, S., F. Casse, M. Chippaux, and M. C. Pascal.** 1975. A mutant of *Escherichia coli* deficient in pyruvate formate lyase. Mol. Gen. Genet. 141:181–184.
39. **Verhoef, C., and P. G. de Haan.** 1966. Genetic recombination in *Escherichia coli*. I. Relation between linkage of unselected markers and map distance. Mutat. Res. 3:101–110.
40. **William, F. R., and L. P. Hager.** 1966. Crystalline flavin pyruvate oxidase from *Escherichia coli*. Arch. Biochem. Biophys. 116:168–176.
41. **Wu, T. T.** 1966. A model for three point analyses of random general transduction. Genetics 54:405–410.

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.